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TITLE: A Medical Research and Evaluation Facility and Studies Supporting the Medical Chemical Defense Program

SUBTITLE: A Comparison of the Biochemical Changes Produced by Vesicating and Non-Vesicating Alkylating Agents in Normal Human Epidermal Keratinocytes

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13. ABSTRACT *(Maximum 200 words)*

Studies were to be conducted under this task to compare alterations in selected biochemical endpoints in human epidermal keratinocytes exposed *in vitro* to either of three vesicating [HD (positive control), mechlorethamine (nitrogen mustard; HN2), and chloroethylethyl sulfide (CEES)] or three non-vesicating alkylating agents [cisplatin, melphalan, and thiotepa (triethylenethiophosphoramide; Thioplex®)]. Stability and solubility data led to use of 200 mg/mL of mechlorethamine in RPMI 1640 medium, cisplatin in multisol, Thioplex® and melphalan in ethanol, and neat solutions of HD and CEES. *In vivo* studies evaluated microblister formation at various HD doses (0.25, 0.50, or 1 μL of neat topical HD) using 8 hairless guinea pigs (HGP). The vesication potential for the alkylating agents was examined in twenty-six HGP. Dosing volumes were streaked over an approximately 1-cm line and consisted of 5 μL for CEES, cisplatin, and Thioplex®, 10 μL for mechlorethamine and melphalan, and 0.5 μL for HD. Statistical evaluations of lesions indicated that mechlorethamine (47 percent with unabraded skin and 100 percent with abraded skin), CEES (100 percent), and HD (100 percent) produced a statistically significant ($p < 0.05$) incidence of microvesication, while Thioplex®, melphalan, and cisplatin did not, even following dosing of abraded skin. These data are in agreement with data on vesicating potential of alkylating chemicals based on ulceration. In June 1996, Task 91-23 was terminated prior to initiating the *in vitro* portion of the study.

14. SUBJECT TERMS
Sulfur Mustard, HD, Vesicating and Non-Vesicating Alkylating Agents, Microvesication, CEES, Melphalan, Cisplatin, Nitrogen Mustard (Mechlorethamine), Thioplex

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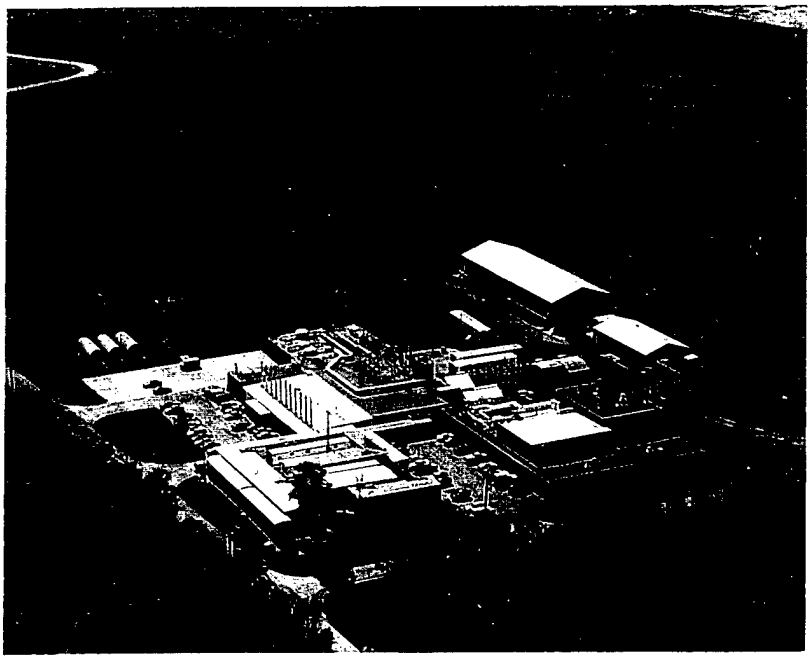

Task 91-23: A COMPARISON OF THE BIOCHEMICAL CHANGES PRODUCED BY VESICATING AND NON-VESICATING ALKYLATING AGENTS IN NORMAL HUMAN EPIDERMAL KERATINOCYTES

To

U.S. Army Medical Research and Development Command

February, 1997

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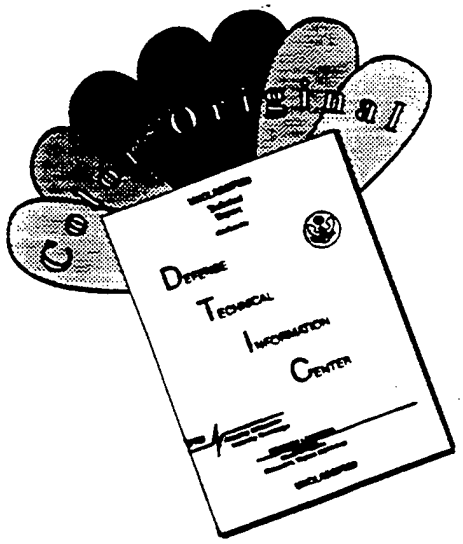

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EXECUTIVE SUMMARY

Task 91-23 was conducted for the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) to compare alterations in selected biochemical endpoints using human epidermal keratinocytes (HEK) exposed *in vitro* to either non-vesicating (e.g., cisplatin) or vesicating (e.g., sulfur mustard; HD) alkylating agents. These data may provide insight into the biochemical mechanism(s) uniquely associated with tissue vesication. Preliminary *in vivo* experiments used three vesicating and three non-vesicating alkylating agents to confirm vesication potential by examining microblister formation in the hairless guinea pig (HGP). The three vesicating, alkylating agents selected were HD (positive control), mechlorethamine (nitrogen mustard; HN2), and chloroethylethyl sulfide (CEES). The three non-vesicating, alkylating agents selected were cisplatin, melphalan, and thiotepa (triethylenethiophosphoramide; Thioplex®). Solubility studies of melphalan, cisplatin, CEES, nitrogen mustard and Thioplex® in compatible solvents were performed at Battelle's Medical Research and Evaluation Facility (MREF) before *in vivo* experiments were initiated. Stability studies of nitrogen mustard, melphulan, and CEES were performed at Battelle's MREF. No ecceptable analytical methods for cisplatin nor thiotepa were established at the MREF during the study for stability determination. American Cyanamid Company (Pearl River, NY) reported Thioplex® (Thiotepa) to be stable for approximately six months. Thioplex® was soluble in ethanol at a concentration of 200 mg/mL. Melphalan was soluble in ethanol at a concentration of 200 mg/mL and did not show significant decomposition (<6 percent) during the course of the study. Cisplatin was not significantly soluble in the Roswell Park Memorial Institute (RPMI) medium, saline, ethanol, Multisol (48.5 percent water, 40 percent propylene glycol, 10 percent ethanol, 1.5 percent benzyl alcohol; v:v), polyethylene glycol 200 (PEG200), or pentane. Cisplatin did form a suspension in PEG 200 and Multisol at 200 mg/mL. CEES was soluble in RPMI at a concentration of 200 mg/mL and did not show significant decomposition (<6 percent) during the course of the study. Nitrogen mustard was soluble in RPMI at a concentration of 200 mg/mL and did not show significant decomposition (<4 percent) during the course of the study.

Eight male HGPs were used to evaluate microblister formation at various HD doses and exposure times. Animals were anesthetized with intramuscular (IM) injections of xylazine hydrochloride and ketamine hydrochloride. Additional injections of xylazine and ketamine at the initial dosage rates were administered IM as needed to maintain anesthesia. Six dose sites (approximately 2 cm by 2 cm) were delineated on the back of each animal using an indelible-ink marker. At three of the sites, HD was applied as an approximately 1 cm streak to deliver either 0.25, 0.50, or 1 μL of neat HD. One site was not dosed with HD and served as a control. The two remaining dose sites were used to determine the effects of the decontamination process. Skin from these two sites were placed in hexane and the concentration of extractable HD determined. There was extensive epidermal and follicular necrosis in all HD-induced lesions, regardless of the dose. There did not appear to be a difference between microblister formation in the center of the lesion and along the edge of the lesion. Fewer microblisters occurred in the high HD dose group, perhaps due to rapid necrosis of epidermal and dermal layers, including capillary cells and resident leukocytes, which could have decreased the inflammatory cell response. The results indicated that liquid HD, when applied as a 1 cm streak, would consistently produce microvesication at all dose levels tested. The 0.50 μL HD dose was used as the positive control in studies comparing vesicating and non-vesicating alkylating agents.

The comparative vesicating and non-vesicating agents dosing experiments evaluated chemicals on abraded and non-abraded skin. A total of 29 male HGPs were used, with histological evaluations performed on skin samples from 26 of these animals. A seven-site dosing-grid was drawn on the dorsum of each animal with each site measuring approximately 2 cm by 2 cm. Compound dosing sites, except for three controls, were randomized and the skin either abraded (when compound penetration was questionable) or not abraded. Compounds were formulated at 200 mg/mL in the following solvents: mechlorethamine in RPMI 1640 medium, cisplatin in multisol, Thioplex® and melphalan in ethanol, or used as neat solutions (HD and CEES). Doses were streaked over an approximately 1-cm line. Dosing volumes consisted of 5 μL for CEES, cisplatin, and Thioplex®, 10 μL for mechlorethamine and melphalan, and 0.5 μL for HD. Two hr after dosing, each site was decontaminated, and

the animals placed individually in polycarbonate cages within the fume hood. Approximately 24 hr after dosing, the animals were euthanatized following deep anesthesia with halothane and by creation of a pneumothorax. The skin samples for histopathology were excised and prepared for examination. Lesions were evaluated for the presence/absence of the following histopathological markers: intracellular edema, epidermal necrosis, pustular epidermitis, microblisters, and follicular necrosis. Statistical evaluations indicated that mechlorethamine (47 percent with unabraded skin and 100 percent with abraded skin), CEES (100 percent), and HD (100 percent) produced a statistically significant ($p < 0.05$) incidence of microvesication, while Thioplex®, melphalan, and cisplatin did not produce a significant incidence, even following exposures of abraded skin. The data presented in this report are in agreement with data classifying the vesicating potential of alkylating chemicals on the basis of ulceration. These alkylating chemicals should provide a useful tool for assessing differences in the mode of cytotoxicity of nonvesicating and vesicating alkylating chemicals and perhaps provide insight into different modes for treating percutaneous HD exposure. On June 4, 1996 a letter was received from the contracting officer of USAMRICD, Daniel Signore, to terminate Task 91-23 prior to initiating the *invitro* portion of the study. This modification of the program prevented experimentation with selected biochemical endpoints.

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A COMPARISON OF THE BIOCHEMICAL CHANGES PRODUCED BY VESICATING AND NON-VESICATING ALKYLATING AGENTS IN NORMAL HUMAN EPIDERMAL KERATINOCYTES

1.0 INTRODUCTION

The U. S. Army Medical Research and Materiel Command (USAMRMC) performs research to characterize the pathophysiology produced by sulfur mustard (HD) in order to design effective therapeutic interventions. There are a number of biochemical parameters, such as poly (ADP-ribose) polymerase (PADPRP) activation, nicotinamide adenine dinucleotide (NAD^+) depletion, adenosine triphosphate (ATP) depletion, decreased glucose utilization, decreased lactate production, and protease secretion, which have been associated with HD-induced cellular toxicity.[1, 2, 3, 4, 5, 6, 7,8, 9] HD is bifunctional, i.e., both an alkylating agent and a vesicant.[1, 2, 3, 4, 5, 6] Although HD, like many other alkylating agents, is thought to exert its toxic effects primarily through irreversible binding and damage to deoxyribonucleic acid (DNA) of cells, the mechanism of HD-induced vesication is unknown.[3, 5] As not all alkylating agents are vesicants,[10] a comparison of biochemical events altered by HD with the effect of other vesicating and nonvesicating alkylating agents on these same endpoints might provide insight into the biochemical mechanisms involved in the vesicating process. The identification of such cellular events may provide new directions for developing therapeutic regimens for percutaneous HD exposure.

1.1 Objective

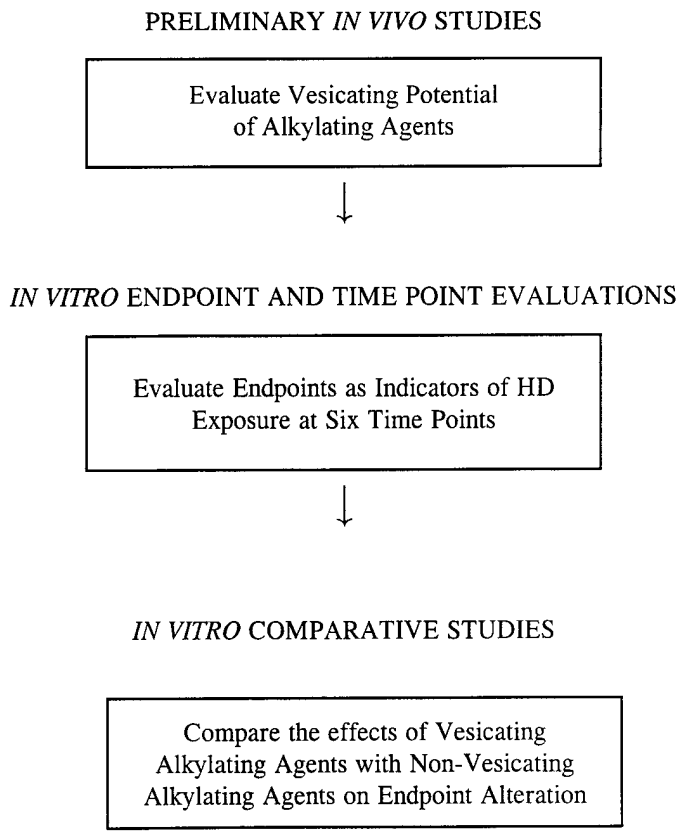
The objective of studies conducted under Protocol 87 (Appendix A) for Battelle's Medical Research and Evaluation Facility (MREF) Task 91-23 was to compare alterations in select biochemical endpoints using human epidermal keratinocytes (HEK) exposed *in vitro* to either non-vesicating[10] or vesicating alkylating agents to provide insight into the biochemical mechanisms uniquely associated with tissue vesication. Three non-vesicating and three vesicating alkylating agents were selected, and to confirm vesicating potential, preliminary *in vivo* studies were conducted to determine the incidence of microblister formation in the hairless guinea pig (HGP). The *in vivo* portion of this task was completed and is presented in this report.

2.0 MATERIALS AND METHODS

This study was conducted at the MREF. Appendix A contains Protocol 87, along with Addenda, Amendments, and Deviation Reports pertaining to this study. Appendix B contains data from chemistry solubility experiments. Appendix C contains data pertaining to effects of HD dose volume, and photomicrographs of skin histopathology. Appendix D contains the Letter Report concerning the effect of HD dose. Appendix E contains data pertaining to the vesicating potential of the six alkylating agents.

2.1 Experimental Design

The study was designed with an *in vivo* portion to confirm the vesicating potential of alkylating agents, and an *in vitro* portion to compare effects on selected biochemical endpoints of vesicating and non-vesicating alkylating agents. A flowchart for the planned *in vivo* and *in vitro* experiments is as follows:



The preliminary *in vivo* experiment was performed to evaluate the incidence of microblister formation at various doses of HD and at various durations of exposure in the HGP. This study was followed by one to evaluate the effect of three vesicating alkylating agents, HD (as positive control), mechlorethamine (nitrogen mustard, HN2), and chloroethylethyl sulfide (CEES), and three non-vesicating alkylating agents, cisplatin, melphalan, and Thioplex® on abraded and non-abraded skin. Task 91-23 was terminated prior to initiating the *in vitro* portion of the study.

2.2 Animal Test System

Male euthymic HGPs, which have been shown to exhibit microvesication (limited dermal-epidermal separation) following percutaneous HD exposure, were used as the model system.[7, 8, 9] A total of 42 animals, weighing between 250 and 361 gm, were purchased from Charles River Laboratories in Portage, Maine. On March 25, 1993, the first nine animals were used to determine an HD dose, and a liquid exposure procedure that would produce consistent microblister formation, and to determine the amount of HD extracted from excised dose sites. In June 1993, Charles River Laboratories personnel informed the MREF Study Director and the Study Attending Veterinarian that their HGP colony was infected with *Listeria*. Charles River eliminated the colony, and this study was delayed until April 1995 when limited numbers (5 to 10) of male HGPs became available. As animals became available, the *in vivo* experiments were completed. A total of 29 male Charles River HGPs were used for the comparative experiments between vesicating and non-vesicating alkylating agents. Histological evaluations were performed on 26 of these animals. Three animals died prior to the 24-hr evaluation time.

Animals were quarantined for a minimum 7-day period and ears were tagged to retain positive identification during handling and observation. HGPs were housed individually in polycarbonate cages equipped with automatic watering systems. Animal rooms were maintained between 65 and 75 degrees F, 40 to 60 percent relative humidity, with a light/dark cycle of approximately 12 hr each per day using fluorescent lighting. Purina Certified Rodent

Chow pellets were fed as specified in Battelle SOP MREF VII-003. No contaminants that would interfere with the results of the study were known to be present in the feed. Water was supplied from Battelle's water system, and given *ad libitum*. No contaminants that would affect the results of the study were known to be present in the water. During dosing and observation periods, animals were anesthetized and positioned on tie-down boards for exposures. Following recovery from anesthesia, the animals were placed in polycarbonate cages within the hood system overnight. No food was provided during dosing and observation periods, but water was provided *ad libitum*.

Battelle's Animal Resources Facilities have been registered with the U.S. Department of Agriculture (USDA) as a Research Facility (Number 31-21) since August 14, 1967 and are periodically inspected in accordance with the provisions of the Federal Animal Welfare Act. In addition, animals for use in research are obtained only from laboratory animal suppliers duly licensed by the USDA. Battelle's statement of assurances regarding the Department of Health and Human Services (DHHS) policy on humane care of laboratory animals was accepted by the Office of Protection from Research Risks, National Institute of Health (NIH) on August 27, 1973. Animals at Battelle are cared for in accordance with the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" (DHHS Publication No. NIH 78-23), and/or in the regulations and standards as promulgated by the Agricultural Research Service, USDA, pursuant to the Laboratory Animal Welfare Act of August 24, 1966, as amended (P.L. 91-579). Protocols of all experiments using animals are reviewed and approved by Battelle's Institutional Animal Care and Use Committee (IACUC) prior to initiation of the study. The program manager accepts responsibility for the proper care and use of animals in the conduct of the research described in this protocol. On January 31, 1978, Battelle's Columbus Division received full accreditation of its animal-care program and facilities from the American Association for Accreditation of Laboratory Animal Care (AAALAC). Battelle's full accreditation status has been renewed after every inspection since the original accreditation. The MREF is a part of the facilities granted full accreditation.

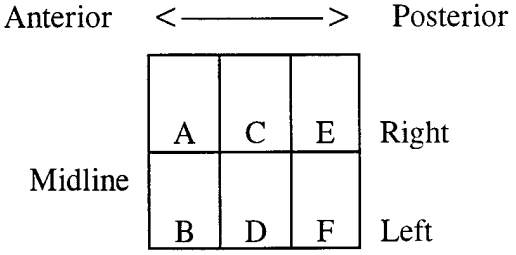
2.3 Test Articles

HD, along with data on lot identification and purity, was obtained trough the U. S. Army Medical Research Institute of Chemical Defense (USAMRICD). Cisplatin, melphalan, and mechlorethamine were purchased from Sigma Chemical Company (St. Louis, MO), and CEES was obtained from Aldrich Chemical Company (Milwaukee, WI). Thioplex®, thiotepa, was purchased from American Cyanamid Company (Pearl River, NY). Identity and purity information on purchased compounds were provided on Material Safety Data Sheets and analyses were not performed by Battelle. Solubility and stability analyses of melphalan, cisplatin, CEES, mechlorethamine and Thioplex® were performed at the MREF and data are in Appendix B.

2.4 Experimental Methods

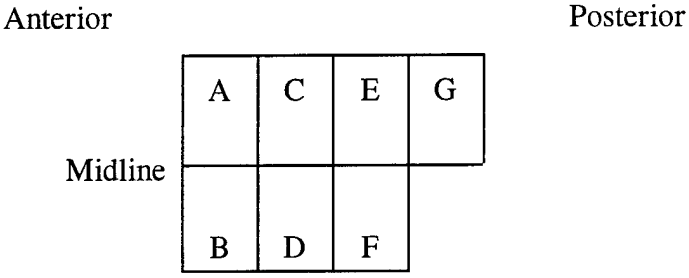
Animal Preparation - The day prior to study, HGPs were weighed and those weighing between 250 and 361 gm were selected for study. Their backs were gently cleaned with isopropanol. On the day of study, the animals were anesthetized by IM injection of approximately 35 mg ketamine hydrochloride/kg BW and approximately 6 mg xylazine hydrochloride/kg BW. On December 13, 1995, after consultation with the USAMRICD veterinary staff, anesthetic doses were modified to IM administration of 13 mg xylazine/kg followed within five min by 87 mg ketamine/kg. Anesthetized animals were secured to a tie-down board in sternal recumbency using either Vet Wrap® or surgical tape about the legs.

Preliminary HD Dose and Experimental Procedure Evaluation - A dosing grid, as shown below, was applied to the dorsum of each animal using an indelabile-ink pen.



Each of the six dose sites measured approximately 2 by 2 cm. The compound dose site for each animal was randomized using a block design. HD was applied as an approximately 1 cm streak. Four of the dose sites were dosed with either 0, 0.25, 0.50, or 1 μL of neat HD. The two remaining dose sites were dosed with 0.0 or 1.0 μL of neat HD and used to establish the effectiveness of the decontamination process. These latter two dose sites were excised and placed in hexane and the concentration of HD extracted determined.

Dosing of Vesicating and Non-Vesicating Alkylating Agents - Due to a limited supply of animals, from 4 to 8 animals were dosed per day. Animals were anesthetized and boosters of xylazine (6 mg/kg) and ketamine (30 mg/kg) were administered IM as needed to maintain anesthesia. A seven-site dosing-grid was drawn on the dorsum of each animal's back and lettered A through G as shown below. Each dose site measured approximately 2 cm by 2 cm.



Compound dose sites for each animal were randomized with the exception of sites E, F, and G and four compounds tested per dosing day. Thiotepa was not available for dosing until late December 1995. Site E was not dosed and was either abraded or not abraded (negative control). Site F was dosed with 0.5 μL of neat HD (positive control). At site G, 5 μL of ethanol was applied to abraded or non-abraded skin (vehicle control). Abraded dose sites were abraded using a 23 ga Becton Dickinsen needle to incise the stratum corneum. Compounds were formulated at 200 mg/mL in the following solvents: mechlorethamine in RPMI 1640 medium, cisplatin in multisol, Thioplex® and melphalan in ethanol, or used as neat solutions (HD and CEES). Doses were streaked over an approximately 1-cm line. Dose volumes were 5 μL for CEES, cisplatin, and Thioplex®, 10 μL for mechlorethamine and melphalan, and 0.5 μL for HD. Exposures were performed on abraded skin when skin penetration of a

compound was questionable.

After Dosing Care - Two hr after dosing, each site was wiped with a dry Q-tip followed by two gentle wipes with Q-tip swabs soaked in 0.5 percent sodium hypochlorite, and two rinses with water soaked Q-tips. The animals were removed from tie-down boards and placed individually in polycarbonate cages equipped with watering bottles within the fume hood for the remainder of the 24-hr exposure period. An Elizabethan collar made of cardboard was placed around each animal's neck to prevent mutilation of the dosed sites.

Biopsy of Dosed Sites - Approximately 24 hr after dosing, the animals were euthanatized by deep anesthesia with halothane followed by incision of the diaphragm to create a pneumothorax. The entire skin of the dorsum was excised using a sharp pair of scissors, a scalpel or a combination, then each dose site was sectioned. Each identified skin section was placed into a specimen cassette which had been labeled with animal number and dose site letter. The cassettes were immediately submerged in 10 percent neutral buffered formalin. After 24 hr, the two specimens exposed for HD extraction purposes were removed and the amount of HD extracted with hexane was determined analytically. As no HD could be detected in the tissue extract or in the formalin, it was considered safe to submit the samples for histopathology. The specimens were submitted for histopathological evaluation after being submerged in the neutral buffered formalin solution for five days.

Histopathological Evaluation - The samples were trimmed in an anterior-to-posterior direction through the thickest portion of the lesion, if grossly discernable. Adjacent normal skin was left attached to each sample for comparison. Trimmed samples were routinely processed for paraffin embedding using alcohol and xylene in a Miles Scientific (Elkhart, IN) VIP 2000/3000 Automatic Tissue Processor or Fisher Histomatic Tissue Processor Model 166A (Pittsburgh, PA). The paraffin-embedded tissues were cut in five micron thick sections, stained with hematoxylin and eosin, and examined microscopically. Lesions were evaluated for the presence/absence of various histopathological markers:

- Intracellular Edema (ballooning degeneration, hydropic degeneration, vacuolar degeneration) of the Epidermis - Characterized by increased cell size, cytoplasmic pallor, and displacement of the nucleus to the periphery of affected cells; refers to all layers of the epidermis.

- Epidermal Necrosis - Primarily refers to the nuclear morphology of those cells in the epidermis; includes condensation and shrinkage (pyknosis), fragmentation (karyorrhexis), and dissolution (karyolysis) of the nucleus. Basal cells are the cells most affected by HD.

- Pustular Epidermitis - The presence of neutrophils within the epidermal layer; under normal conditions and without the appropriate inflammatory mediator release, there should be no neutrophils present.

- Microblister - A separation and loss of attachment (visible at the light microscope level, i.e., 400x) of the basal cell layer from the underlying basement membrane (not visible with routine staining). At a minimum, a microblister must represent the loss, or dissolution, of two adjacent basal cells. Frequently within this newly created space there will be cellular debris, neutrophils, and/or macrophages.

- Follicular Necrosis - Necrosis of the basal cell layer and other epidermal layers which invaginate into the dermis and line the hair follicles.

The severity of the markers were graded from one to four, with four being the most severe. The specimens were evaluated without knowledge of the actual exposures.

2.5 Statistical Evaluation

A statistical evaluation of the incidence of microvesication was conducted by comparing treated sites with negative control sites within each animal. Mechlorethamine, CEES, and HD treated sites were compared to the untreated control site with unabraded skin. Cisplatin, melphalan, and Thioplex® treated sites were compared to the ethanol control site with abraded skin. Mechlorethamine was compared to the RPMI control site with abraded skin. Statistical comparisons were made using McNemar's test for paired data with response/no response outcomes. Comparisons were made using only those animals that received both treatments.

3.0 RESULTS

Preliminary HD Dose Evaluation and Experimental Procedure Evaluation - In all guinea pigs, the vehicle control site had negligible pathology. The skin was essentially normal

except for isolated necrotic basal cells which occurred in six of the eight animals. At the HD-treated sites, the affected epidermis was uniformly and diffusely necrotic, having the appearance of a thermal or chemical burn. At these affected sites the epidermis varied in appearance only slightly, due mainly to the degree of pyknosis of basal or other epidermal cells, the degree of neutrophil infiltration, and the degree of separation of basal cells from the underlying dermis. The scores for these evaluations are provided in Appendix C. Representative photomicrographs of lesions are provided in Attachment C of Appendix D. A summary of the histopathology is provided in Table 1.

TABLE 1. SUMMARY OF HISTOPATHOLOGY FOR EACH DOSE OF SULFUR MUSTARD

		HD Dose (μL)			
		0	0.25	0.5	1
Intracellular Edema	Mean	0.0	1.1	1.0	1.0
	STD	0.0	0.4	0.5	0.0
Epidermal Necrosis	Mean	0.8	3.9	3.6	3.3
	STD	0.5	0.4	0.7	0.7
Pustular Epidermitis	Mean	0.1	1.5	1.4	1.1
	STD	0.4	1.1	1.2	1.1
Microblister	Mean	0.0	3.3	3.1	1.9
	STD	0.0	0.7	0.6	0.6
Follicular Necrosis	Mean	0.0	3.6	3.9	3.9
	STD	0.0	0.7	0.4	0.4

Dosing of Vesicating and Non-Vesicating Alkylating Agents - The percent incidence of microvesicle formation due to exposure of unabraded skin to alkylating agents are presented graphically in Figure 1. The incidence of microvesicle formation on unabraded skin was

significantly different ($p < 0.05$) from the control site for mechlorethamine (47 percent), CEES (100 percent), and HD (100 percent). Since cisplatin and melphalan did not produce microvesication or other signs of pathology following exposure of intact skin (data not shown), the dosing sites were abraded prior to exposure to facilitate compound penetration. Thioplex®, reported to be a non-vesicating alkylating agent, was dosed only onto abraded skin to provide the best opportunity for producing dermal lesions. Due to the partial response observed with mechlorethamine, abraded dosing sites were also exposed to mechlorethamine to assess the response. Percent microvesicle incidence for abraded skin exposures are presented in Figure 2. When the skin was abraded, the incidence of mechlorethamine-induced microvesication increased from 47 percent to 100 percent. Similar to the response observed with unabraded skin, Thioplex® and cisplatin did not cause a significant ($p < 0.05$) incidence of microvesicle formation on abraded skin. The lesions were evaluated for severity using a semi-quantitive scale ranging from 0 to 4, where 0 is absent and 4 is severe. The average severity scores for microvesication were 3.8 for HD, 3.9 for CEES, and 1.7 for mechlorethamine (abraded skin exposures). Data are presented in Appendix E.

4.0 CONCLUSIONS

Preliminary HD Dose and Experimental Procedure Evaluation - Percutaneous liquid HD exposures produced similar effects across the dose levels examined. At all HD dosed sites there was extensive epidermal and follicular necrosis regardless of dose. Microblisters were observed in all lesions, and were found anywhere along the site where the epidermis was necrotic. There did not appear to be a difference between microblister formation at the center or edge of the lesion. Fewer microblisters occurred in the high HD dose group. This could have occurred due to rapid effects on the epidermal and dermal layers, including capillary and resident leukocyte toxicity, which could have blunted inflammatory cell response with edema and microblister formation. The results of this experiment indicated that liquid HD, when applied as a 1-cm streak, could consistently produce microvesication at all dose levels tested.

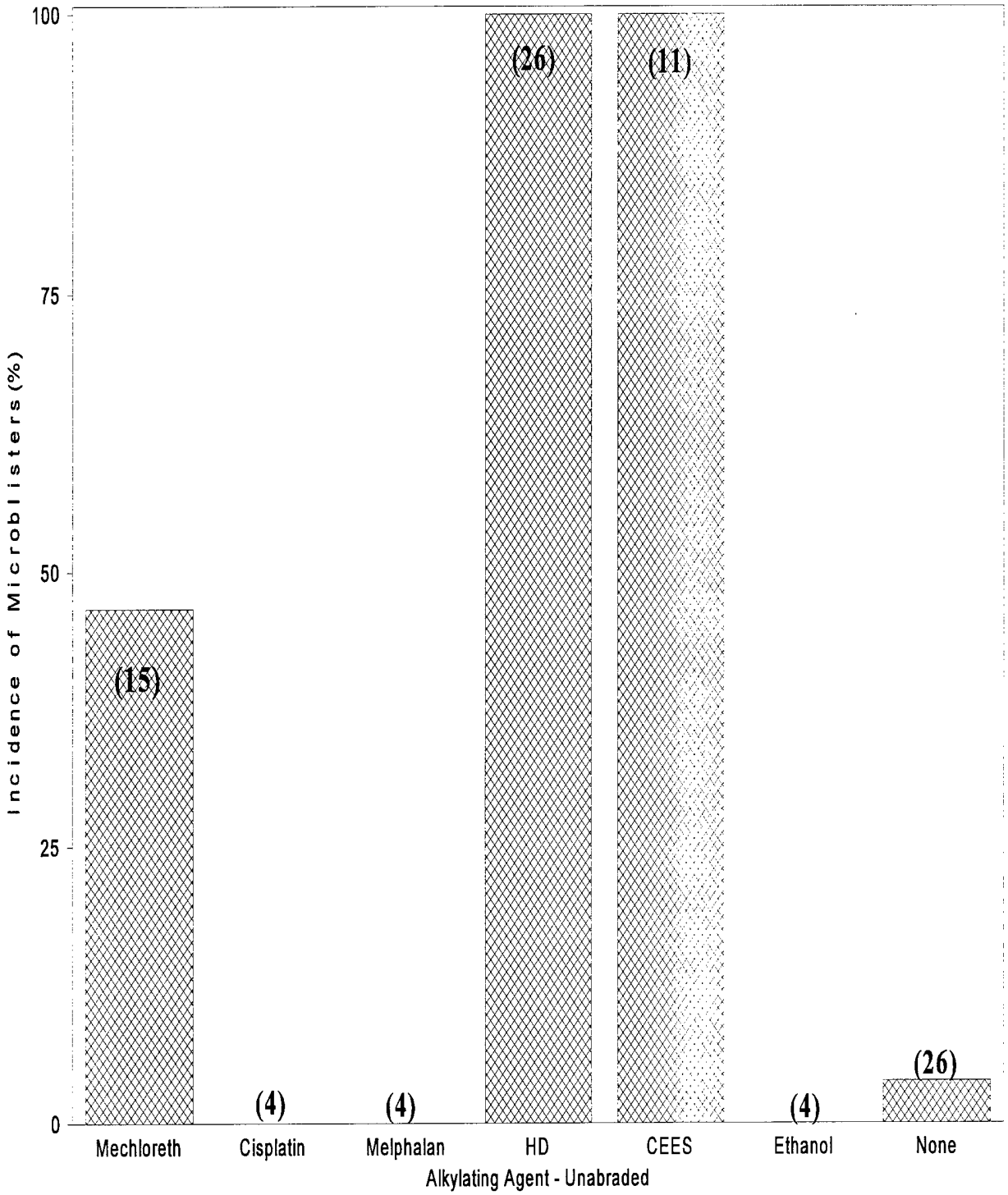


Figure 1. Percent Incidence of Microblisters for Unabraded Surface

Parenthesis ( ) indicates sample size for each experimental group.

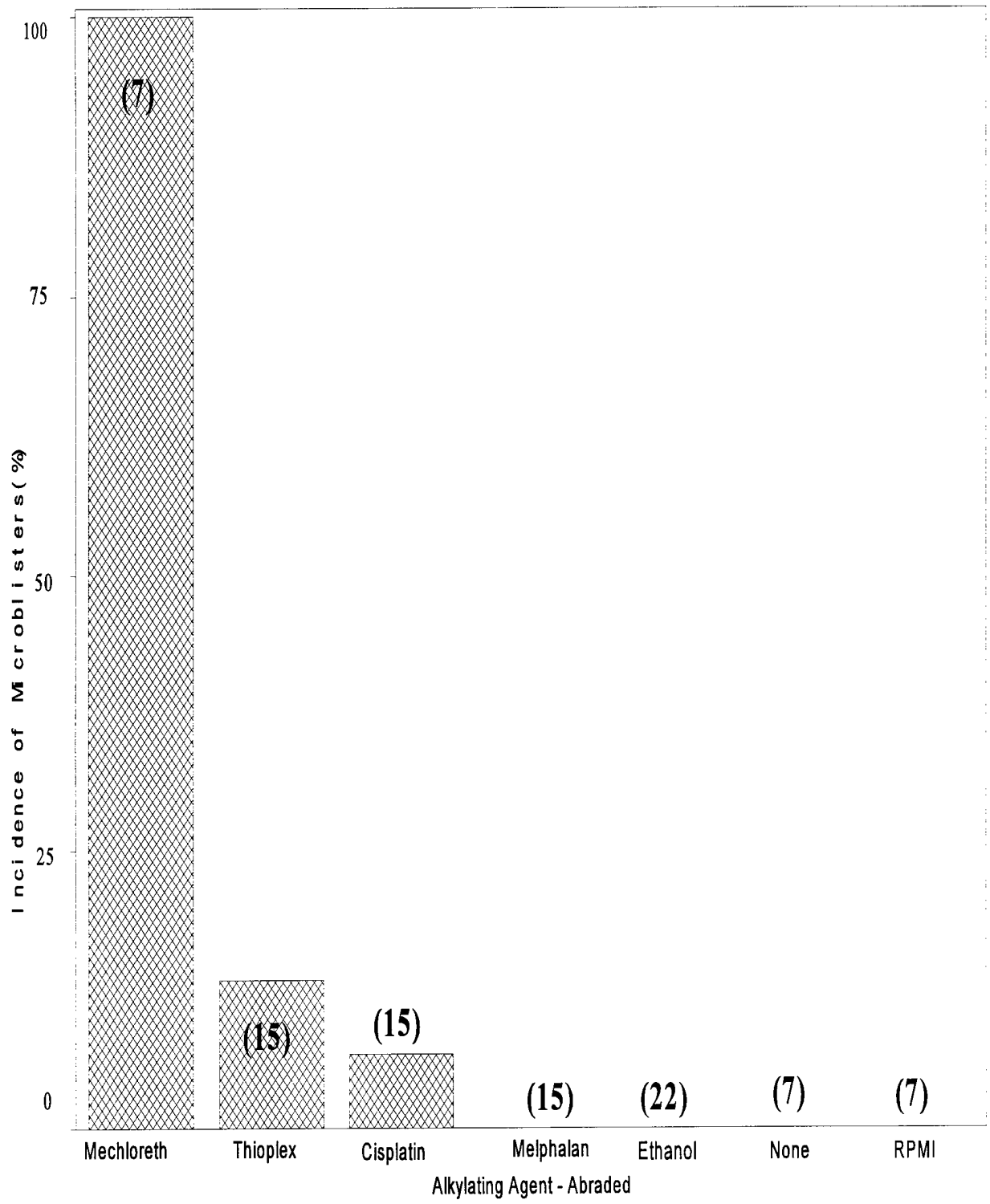


Figure 2. Percent Incidence of Microblisters for Abraded Surface

Parenthesis ( ) indicates sample size for each experimental group.

When a new animal colony was developed, 0.50 μL HD was used as the positive control dose for alkylating agent dosing experiments.

Dosing of Vesicating and Non-Vesicating Alkylating Agents - Data in the literature indicate that DNA alkylating compounds such as cisplatin, melphalan, and thiotepa do not cause ulceration in the mouse following intradermal administration.[10] HD-induced microvesication in HGP has been demonstrated,[7, 8, 9] and in the present studies, HD was used as a positive control for microvesication. Statistical evaluations indicated that mechlorethamine (47 percent with unabraded skin and 100 percent with abraded skin), CEES (100 percent), and HD (100 percent) produced a statistically significant ($p < 0.05$) incidence of microvesication, while Thioplex®, melphalan, and cisplatin did not produce a significant incidence, even when applications were made to abraded skin. The data presented in this report are in agreement with previous data classifying the vesicating potential of alkylating chemicals on the basis of ulceration. For water-soluble, non-vesicating, alkylating agents, abrading the skin to facilitate penetration is recommended. These alkylating chemicals should provide a useful tool for assessing differences in the mode of cytotoxicity of nonvesicating and vesicating chemicals, and perhaps provide insight into different modes of therapeutic intervention for percutaneous HD exposure.

5.0 ACKNOWLEDGMENTS

This work was supported by the U.S. Army Medical Research and Materiel Command under Contract DAMD17-89-C-9050. Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army. In conducting research using animals, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

6.0 REFERENCES

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APPENDIX A

MREF PROTOCOL 87

A Comparison of the Biochemical Changes Produced by Vesicating and Non-Vesicating Alkylating Agents in Normal Human Epidermal Keratinocytes

Study performed by Battelle Columbus Operations,
505 King Avenue, Columbus, OH 43201-2693

1. Principal Investigator and Manager: David W. Hobson, Ph.D., D.A.B.T., Medical Research And Evaluation Facility (MREF)

2. Study Director: James A. Blank, Ph.D.

3. Study Veterinarian: Allan G. Manus, D.V.M.

4. Sponsor: U.S. Army Medical Research and Development Command (USAMRDC)

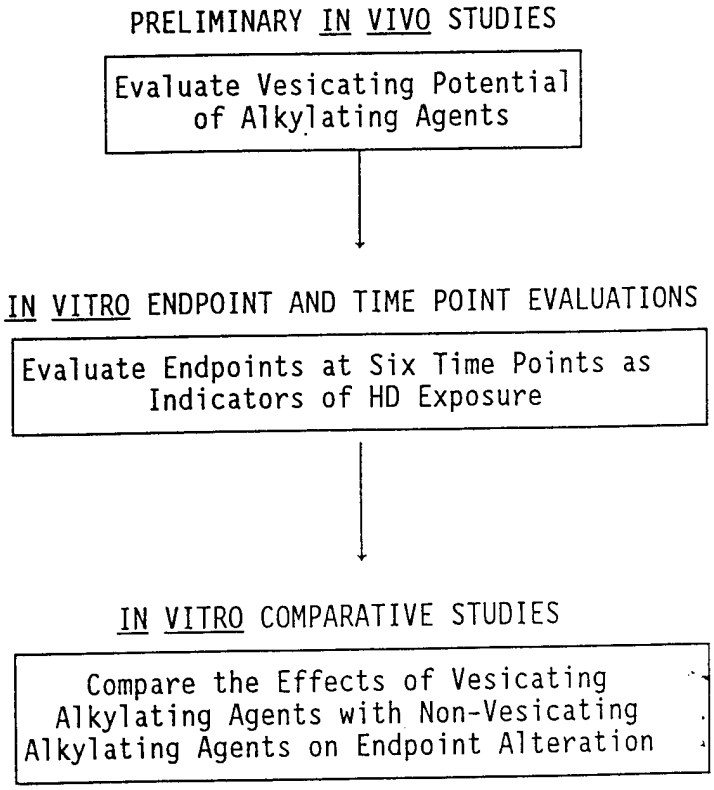
5. Sponsor Monitor: LTC Don W. Korte, Jr., U.S. Army Medical Research Institute of Chemical Defense (USAMRICD)

6. Background: The USAMRDC is performing research to characterize the pathophysiology produced by sulfur mustard (HD), in order to design effective therapeutic interventions. There are a number of biochemical parameters, such as poly(ADP-ribose) polymerase (PADPRP) activation, nicotinamide adenine dinucleotide (NAD^+) depletion, adenosine triphosphate (ATP) depletion, decreased glucose utilization, decreased lactate production, and protease secretion, which have been associated with sulfur mustard (HD)-induced cellular toxicity. HD is a bifunctional alkylating agent which possesses vesicating properties. Although HD, like many other alkylating agents, is thought to exert its toxic effects primarily through irreversible binding and damaging of the deoxyribonucleic acid (DNA) of cells, the mechanism of HD-induced vesication is unknown. As not all alkylating agents are vesicants, a comparison of biochemical events altered by HD with the effect of other vesicating and nonvesicating alkylating agents on these same endpoints, may provide insight into the biochemical mechanism(s) involved in the vesicating process. The identification of such cellular events may provide new directions for developing therapeutic regimens for percutaneous HD exposure.

7. Objective: The objective of studies conducted under this protocol for MREF Task 91-23, "A Comparison of the Biochemical Changes Produced by Vesicating and Non-Vesicating Alkylating Agents in Normal Human Epidermal Keratinocytes", is to examine various biochemical endpoints *in vitro* using human epidermal keratinocytes (HEK) exposed to either non-vesicating (e.g., cis-platinum, melphalan, thiotepa) or vesicating (e.g., HD, chloroethyl sulfide, mechlorethamine) alkylating agents in order to provide a comparative dataset from which biochemical endpoints uniquely associated with tissue vesication may be identified.

8. Experimental Overview: For these studies, three non-vesicating alkylating agents, and three vesicating alkylating agents will be used for the comparative studies. To confirm the vesicating potential of the alkylating agents selected for use in the in vitro comparative tests, preliminary in vivo studies examining microblister formation in hairless guinea pigs will be performed. Final selection of the alkylating agents for the comparative in vitro studies will be made in conjunction with the USAMRICD Technical Point of Contact (POC), Task Area Manager (TAM), and Contracting Officer's Representative (COR), following completion of the preliminary in vivo tests.

Preliminary in vitro studies with HD will be performed to identify endpoints which respond strongly to HD exposure and to establish the time points at which these responses occur. These preliminary studies will provide an HD dataset for inclusion in the comparison of the alkylating agents and will serve to minimize the number of endpoints and time points used for subsequent comparative in vitro studies involving the other five alkylating agents. In vitro comparative studies will then be performed with two vesicating alkylating agents and three non-vesicating alkylating agents to examine for differences in endpoint responses between the two types of alkylating agents. The endpoints and time points used for the in vitro comparative studies will be selected in conjunction with the POC, TAM, and COR. A flowchart for the in vivo and in vitro studies is as follows:



9. Experimental Design:

A. Animal Test System

(1) Animals - Male euthymic, hairless guinea pigs supplied by Charles River Laboratories weighing between 250 to 350 gm will be used for this study. Hairless guinea pigs were chosen because microvesication or epidermal-dermal separation has been shown to occur in these animals following percutaneous HD exposure.

(2) Initial Weight - Guinea pigs used for these studies will weigh between 250 and 350 gm.

(3) Quarantine - Animals are quarantined for a minimum 7-day period. Guinea pigs in good physical condition are then weighed and randomized into weight-homogenized treatment groups.

(4) Acclimation - If animals are not quarantined at the MREF, then all animals will be held at the MREF for at least 24 hr prior to study initiation.

(5) Animal Identification - All animals are ear tagged to retain positive identification during animal handling and observation.

(6) Housing - Animals are housed individually in polycarbonate cages equipped with automatic watering systems.

(7) Lighting - Fluorescent lighting, with a light/dark cycle of 12 hr each per day.

(8) Temperature - Maintained at 60 F (± 5 F).

(9) Humidity - Maintained at 50 percent (± 10 percent).

(10) Diet - Purina Certified Rodent Chow pellets are available as described in Battelle SOP MREF VII-003. No contaminants are known to be present in the feed that would interfere with the results of the study.

(11) Water Supply - Water is supplied from the public water system, and given ad libitum during quarantine and holding. No contaminants are known to be present in the water that would affect the results of the study.

(12) Animal Care During Test - All animals are positioned on tie-down boards for compound exposures and are retained in polycarbonate cages within the hood system during the treatment and observation

periods. No food is provided during the treatment and observations periods, but water is provided ad libitum.

(13) Laboratory Animal Welfare Practices - Battelle's Animal Resources Facilities have been registered with the U.S. Department of Agriculture (USDA) as a Research Facility (Number 31-21) since 14 August 1967 and are periodically inspected in accordance with the provisions of the Federal Animal Welfare Act. In addition, animals for use in research are obtained only from laboratory animals suppliers duly licensed by the USDA. Battelle's statement of assurances regarding the Department of Health and Human Services (DHHS) policy on humane care of laboratory animals was accepted by the Office of Protection from Research Risks, National Institute of Health (NIH) on 27 August 1973. Animals at Battelle are cared for in accordance with the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" (DHHS Publication No. NIH 78-23), and/or in the regulations and standards as promulgated by the Agricultural Research Service, USDA, pursuant to the Laboratory Animal Welfare Act of 24 August 1966, as amended (P.L. 91-579). Protocols of all experiments using animals are reviewed and approved by Battelle's Institutional Animal Care and Use Committee (IACUC) prior to initiation of the study. The program manager accepts responsibility for the proper care and use of animals in the conduct of the research describe in this protocol.

(14) Accreditation - On 31 January 1978, Battelle's Columbus Division received full accreditation of its animal-care program and facilities from the American Association for Accreditation of Laboratory Animal Care (AALAC). Battelle's full accreditation status has been renewed after every inspection since the original accreditation. The MREF is a part of the facilities granted full accreditation.

B. Cellular Test System

(1) Normal human epidermal keratinocytes (HEKs) will be the target cell type used for these studies. These cells are of human origin and are one cell type encountering HD during percutaneous exposure.

(2) Tertiary passaged HEK cultures which had been seeded at 40,000 to 50,000 cells per square cm of growth area and are approximately 75 percent confluent will be used for these studies. The cells are received as primary cryopreserved HEKs and will be expanded as secondary cultures in tissue culture flasks (T-flask; 6 - 30 square cm flask per HEK cryopreserved ampule) prior to being passaged as tertiary cultures in microtest plates.

(3) HEKs cultures will be maintained at 37 C (± 0.5 C), 5 percent carbon dioxide (± 0.5 percent) in air mixture, and a saturated relative humidity atmosphere.

C. Test Material - Chemical surety materiel (CSM) will be supplied by USAMRICD. Purity, appropriate identification (batch number, lot number, state), stability data, and material safety data sheets will be supplied by USAMRICD.

(1) Purity and stability are confirmed periodically by the MREF for CSM stored at the MREF.

(2) Surety, security, and safety procedures for the use of CSM are thoroughly outlined in facility plans, in personnel requirements for qualifications to work with CSM, and in standard operating procedures for the storage and use of CSM.

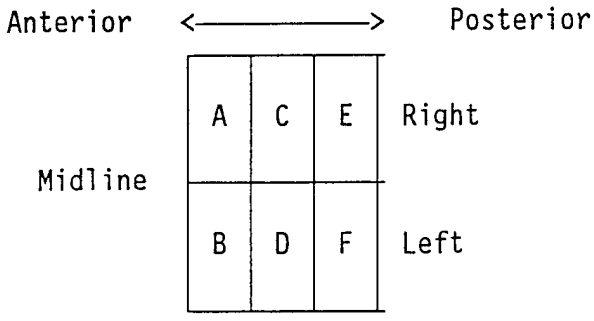
10. Preliminary In Vivo Testing - This test is performed to assess the vesicating potential of alkylating agents when percutaneously applied at 24 hr post exposure. The extent of microblister formation is determined by histopathologic examination of exposed tissue.

A. Animal Preparation for Testing -

(1) Randomization - The day prior to the study, animals are weighed and randomized into weight homogenized treatment groups. The animal backs are cleaned with ethanol or isopropanol.

(2) Anesthetization - Guinea Pigs are anesthetized by intramuscular (i.m.) injection of ketamine (30 mg/kg) and xylazine (6 mg/kg). Booster injections of ketamine (30 mg/kg) and xylazine (6 mg/kg) will be given as needed.

(3) Marking of Test Sites - Animals are anesthetized per Section 10.A.(1) then placed in sternal recumbency with all legs taped to a tie-down board. An application site/dosing grid as shown below is applied to the dorsum of each animal using a felt-tipped pen.



Each dose site will contain either a different alkylating agent or a different dose of the same alkylating agent. The "E" site will contain the negative control (no dose) and the "F" site will contain the positive control (HD dose).

B. Alkylator Agent Exposures - Compounds to be evaluated for vesication will be maximally solubilized in an appropriate solvent and applied as a liquid exposure. The alkylating agents are applied as a 1 cm streak and will be delivered using a Hamilton microliter syringe. The animals will be kept in individual polycarbonate cages equipped with watering bottles contained within the fume hood for the remainder of the 24-hour exposure period.

C. Biopsy of Dosed Sites - At the end of the 24 hr exposure period, the animals are euthanitized by over exposure to halothane. Halothane saturated gauze is placed in the bottom of a bell jar, the halothane is allowed to vaporize, then the animals are placed in the jar. The skin sections are excised and placed in a container of 5 percent neutral buffered formalin. The samples must sit in the neutral buffered formalin solution for 24 hr before being submitted for histopathologic examination.

11. HD Evaluation Studies - Preliminary in vitro studies will be performed with HD to identify cellular and biochemical endpoints which respond most strongly to HD challenge and to aid in selecting the time points at which these endpoints will be examined in the subsequent in vitro comparative studies. These studies consist of an HD concentration response to establish the relationship between HD concentration and HEK viability at 24 hr post exposure and a time-course studies using fixed HD concentrations to evaluate the impact of HD on the endpoints listed in Table 1.

A. HD Concentration Response Study - A concentration response study will be performed to establish the relationship between HD concentration and cellular viability at 24 hr post exposure. Viability will be measured using a propidium iodide exclusion method as described in Section 13.A. This study will utilize eight HD concentrations and will be repeated two times to obtain good estimates of the fixed HD challenge concentrations for use in subsequent time-course studies. Each experimental group will consist of triplicate observations. From these data, the HD concentrations that decrease cellular viability by 25 percent (IC_{25}), 50 percent (IC_{50}), and 75 percent (IC_{75}) will be estimated and used as the fixed HD challenge concentrations in the time-course studies described below.

B. Time-Course Study - These studies examine the endpoints listed in Table 1 at six different times following exposure to each of the fixed HD challenge concentrations as well as a vehicle (tissue culture

medium) control. Each experimental group will consist of triplicate observations and the study will be repeated two times. The concentration and time-course studies will produce an approximate 2500 datapoints (Table 1).

TABLE 1. TECHNICAL DETAIL FOR CONCENTRATION RESPONSE AND TIME-COURSE STUDIES

Study Type	Number Alkylating Agents	Number Treatment Groups[a]	Number Time Points[b]	Number Observations Per Treatment Group	Total Number Experiments	Data Points Total
Conc. Response	1	8	1	3	3	72
Time Course:						
•Cytometry	1	3	6	3	3	162
•NAD^+	1	3	6	3	3	162
•ATP	1	3	6	3	3	162
•Glucose Utilization	1	3	6	3	3	162
•Lactate Production	1	3	6	3	3	162
•PADPRP Activity	1	3	6	3	3	162
•Prostaglandin E_2	1	3	6	3	3	162
•Leukotriene B_4	1	3	6	3	3	162
•Interleukin-1	1	3	6	3	3	162
•Chromogenic Substrate 1	1	3	6	3	3	162
•Chromogenic Substrate 2	1	3	6	3	3	162
•Chromogenic Substrate 3	1	3	6	3	3	162
•Chromogenic Substrate 4	1	3	6	3	3	162
•Chromogenic Substrate 5	1	3	6	3	3	162
•Chromogenic Substrate 6	1	3	6	3	3	162
					Total	2,502

[a]Number of treatment groups per alkylating agent in addition to the vehicle control.
[b]Number of time points at which each treatment group will be examined.

12. In Vitro Comparative Study: In vitro comparative studies will be performed to assess whether differences in biochemical endpoint alterations as a function of non-vesicating versus vesicating alkylating agent are observed. These studies will examine the effect of three non-vesicating alkylating agents and two additional vesicating alkylating agents on endpoint alterations. The endpoints and time points used in these experiments are dependent upon the studies performed in Section 11.B. The endpoints and time points will be selected in conjunction with the USAMRICD COR, TAM, and POC.

A. Concentration Response Study - A concentration response study will be performed to establish the relationship between alkylating agent concentration and cellular viability at 24 hr post exposure. This study will utilize eight alkylating agent concentrations and will be repeated two times to obtain good estimates of the fixed challenge concentrations for use in subsequent time-course studies. Each

experimental group will consist of triplicate observations. From these data, the alkylating agent IC_{25}, IC_{50}, and IC_{75} are estimated for use as the fixed challenge concentrations in the time-course experiments.

B. Time-Course Study - These experiments consist of examining the endpoints selected from the studies involving HD at three time points following exposure to each of the fixed HD challenge concentrations as well as a vehicle control. The three time points at which all endpoints will be evaluated are selected in conjunction with the USAMRICD POC and COR based upon the studies performed with HD. Each experimental group will consist of triplicate observations and the study will be repeated two times.

13. Endpoint Measurement:

A. Cell Viability - Battelle SOP MREF V-001 and MREF Method/In Vitro No. 15 will be followed for cell viability measurements as applicable. Current methods are for measuring mononuclear leukocyte cell viability and, therefore, a new MREF method or modification of an existing MREF and USAMRICD method will be required for evaluation of this adherent cell type in a 96-well plate format. The procedure developed will utilize the following procedure:

(1) Cellular viability will be measured by flow cytometer assessing propidium iodide incorporation into nonviable cells. At the designated time, the tissue culture supernatant is transferred to a second vessel and trypsin is added to the cultures to detach the adherent cells.

(2) Once the cells have detached, trypsin neutralizing solution is added to neutralize the action of the trypsin and the entire mixture is combined with the original tissue culture supernatant.

(3) The cells are pelletized, and the majority of the supernatant is aspirated. Propidium iodide is added and the tube gently mixed, incubated for at a minimum of 5 min, then analyzed using a flow cytometer.

B. NAD^+ - NAD^+ will be measured following methodology contained in Battelle SOP MREF V-003 and MREF Method/In Vitro No. 1. The method may need to be modified so as to measure ATP from the same cellular digest.

C. ATP - ATP measurements will be made using a bioluminescent assay. ATP measurements are made on the cellular extracts used for NAD^+ analyses. Current cellular digestion procedures as outlined in MREF Method/

In Vitro No. 1 may require modification so as to use the same extract to assay ATP and NAD^+.

D. PADPRP Activity - PADPRP can modify proteins by attaching the ADP-ribose moiety of NAD^+ to the protein. PADPRP activity is measured following methodology that will be developed during the course of the task by Battelle and USAMRICD investigators. Current USAMRICD procedures use radiolabeled NAD^+ and measure incorporation of label into proteins. In order to make the assay more economical, an attempt will be made to adapt this assay to a 24-well plate system and, if possible, to a 96-well microplate system.

E. Glucose Utilization and Lactate Production - The level of glucose and lactate in the tissue culture supernatant will be assessed using a Biochemical Analyzer made by YSI, Inc. This analyzer can make rapid measurements of glucose and lactate using immobilized enzyme electrode technology.

F. Protease Activity - Tissue associated protease activity will be measured using a series of different chromogenic substrates. Protease activity is measured spectrophotometrically. The commercially available chromogenic substrates examined are Chromozym TRY, TH, U, PK, TPA, and a Collagenase substrate (PZ-Pro-Leu-Gly-Pro-D-Arg). The parameters for this assay (i.e., concentration of chromogenic substrate and length of incubation) will be defined within this task.

G. IL-1, $PG\text{-}E_2$, and $LT\text{-}B_4$ - The level of these factors in the tissue culture supernatant will be assessed by enzyme immunoassay using commercially available kits.

14. Assay Controls: HD will be used as the assay positive control for the conduct of the alkylating agent time response studies. The dataset used for these purposes is very limited as it is based only upon the HD time-course studies. If the mean value of the positive assay control falls outside three standard deviations of the mean, then the data are considered suspect. The experimental procedures will be reviewed to determine the cause of the positive assay control shift. If the cause of the extreme value cannot be determined, then the influence of this experiment on the final results will be assessed before including or omitting the dataset.

15. Record Maintenance: The following records are to be maintained for MREF Task 91-23:

A. CSM accountability log and inventory,

B. Reagent preparation,

C. Decontamination and disposal records, and

D. Any other records needed to reconstruct the study and demonstrate adherence to this protocol.

16. Reports:

A. Interim letter report covering the conduct of the in vivo studies, the HD concentration and time-course studies will be prepared and submitted.

B. At the end of Task 91-23, a Draft Final Report will be prepared and submitted to USAMRICD within 60 working days of task completion. The Draft Final report includes, at a minimum, the following sections:

(1) Signature page of key study personnel and their responsibilities,

(2) Experimental design,

(3) Test material description

(4) Tabulation and statistical data analysis, and

(5) Discussion and conclusion.

The Final Report will be submitted within 30 days of receiving the Draft Final Report comments from USAMRICD.

17. Approval Signatures:

James A. Blank — 13 October 1992
James A. Blank, Ph.D.
Study Director
Date

David W. Hobson — 15 OCT 92
David W. Hobson, Ph.D., D.A.B.T.
MREF Principal Investigator and Manager
Date

Allan G. Manus — 10/16/92
Allan G. Manus, D.V.M.
Chief Veterinarian
Date

John W. Korte — 10/14/92
LTC Don W. Korte, Jr.
USAMRICD COR
Date

A Comparison of the Biochemical Changes Produced by
Vesicating and Non-Vesicating Alkylating Agents in
Normal Human Epidermal Keratinocytes

Amendment No. 1

Change: Replace Page 6, Section 10.C. with the following

C. Biopsy of Dosed Sites - Animals exposed to HD are handled in accordance with Battelle SOP MREF II-003. At the end of the 24-hr exposure period, the animals are euthanized with halothane in the fume hood. Halothane saturated gauze is placed in the bottom of a bell jar, the halothane is allowed to vaporize, then the animals are placed in the jar. Samples of skin tissue from various test sites are harvested from euthanized animals. Unaffected skin adjacent to a test site is lifted with forceps, and the test site skin is excised with scissors and placed into 5 percent neutral buffered formalin (NBF) solution.

(1) The skin section is excised in the fume hood, the appropriate label attached, then placed into a container of NBF fixative which is held at the hood face. The cap to the fixative is put in place, the container placed in a secondary and then removed from the hood face. Each specimen is identified by placing it into a labeled jar or cassette, or by stapling an identification card to one corner of the specimen. Specimens are identified by task and protocol numbers, charge account number, study director, date and time of sample collection, animal identification number, and treatment site. Where appropriate, warning labels stating that the skin samples were exposed to HD are affixed to the outside of each container and to the outside of the box used for transportation. All HD-exposed samples are retained at the MREF for 24 hrs before being transported to another facility for histologic processing. Standard sectioning with hematoxylin and eosin staining are performed on each sample.

(2) Prior to submitting tissue samples for histopathology, tissue samples which had been exposed to the maximal HD dose (1 μL) are extracted, and HD content is determined analytically to confirm decontamination during the fixative process. In addition, the NBF solution is analyzed to determine whether HD exists after the 24-hr retention period (Battelle SOP MREF III-005). Validating these analytical methods is accomplished in the following pilot study.

(a) Extraction of HD from Dorsal Skin: One anesthetized, restrained hairless guinea pig is dosed with 1 μL of HD at each of five test sites (Battelle SOP MREF II-003). A sixth site serves as a negative control. Two hours later, the six sites are decontaminated with 0.5 percent NaOCl followed by two distilled water rinses (Battelle SOP MREF II-002). The animal is

euthanized, and the dorsal skin is excised and pinned to a cutting board. The six sites are separated with a scalpel, and each is immediately placed into a scintillation vial containing 2 mL of hexane with an internal standard. The individual transferring the tissue dons a clean set of gloves. Each vial is vigorously shaken, then opened and aliquoted into three, 1-mL GC vials for chromatographic analysis for HD (Battelle SOP MREF III-002).

(b) Extraction of HD from NBF Solution (Battelle SOP MREF III-002): A volume of 5 percent NBF solution is spiked with 1 µL of HD, q.s.'d to 100 mL in a 100-mL volumetric, sealed, and inverted several times. The volumetric seal is removed and 10 mL of its contents are aliquoted into each of three 15-mL capacity culture tubes. A 1-mL volume of hexane with internal standard is added to each culture tube, which is then sealed and shaken vigorously. Three, 1-mL volumes of each tube are aliquoted into GC vials for chromatographic analysis for HD.

Reason: Histologic processing and examination are necessary to determine microvesication in hairless guinea pigs. This change describes the handling of specimens which were previously exposed to HD.

Impact: This modification does not have an impact on this study.

Amendment No. 1 Approval Signatures:

James A. Blank, Ph.D.
Study Director — Date: 16 April 1993

David L. Stitcher
CIH, Safety & Surety Officer — Date: 16 April 1993

LTC Don W. Korte, Jr.
USAMRICD COR — Date: 20 APR 93

A Comparison of the Biochemical Changes Produced by
Vesicating and Non-Vesicating Alkylating Agents in
Normal Human Epidermal Keratinocytes

Protocol Amendment No. 2

Change 1: Page 1, Section 1.

Change to: "Co-Principal Investigator and Manager: John B. Johnson, D.V.M., Medical Research and Evaluation Facility (MREF)".

Reason for change:

Since this protocol was last revised, the principal investigator and manager has changed.

Change 2: Page 1, Section 3. Study Veterinarian.

Change to: "Tracy A. Peace, D.V.M.".

Reason for change:

Since this protocol was last revised, the study veterinarian has changed.

Change 3: Page 1, Section 4. Sponsor.

Change to: "U.S. Army Medical Research and Materiel Command (USAMRMC)".

Reason for change:

Since this protocol was last revised, the name of the sponsoring organization has been changed.

Change 4: Page 1, Section 5. Sponsor Monitor.

Change to: "LTC Richard R. Stotts, D.V.M., P.h.D., U.S. Army Medical Research Institute of Chemical Defense (USAMRICD)".

Reason for change:

Since this protocol was last revised, the sponsor monitor has changed.

Approved by:

James A. Blank, Ph.D., D.A.B.T.
Study Director

11/8/95
Date

LTC Richard R. Stotts, D.V.M., Ph.D.
USAMRICD COR

9 NOV 95
Date

A Comparison of the Biochemical Changes Produced by Vesicating and Non-Vesicating Alkylating Agents in Normal Human Epidermal Keratinocytes

MREF Protocol 87. Amendment No. 3

Change Number No. 1

Change: Delete the following statement in section 9.A.(2) Initial Weight at the end of the first sentence.

"250 and 350 gm."

Replace with the following range.

"Animals will weigh 250 gm at a minimum".

Reasons for Changes:

The supplier of the hairless guinea pigs, Charles River Laboratories, has had difficulty providing animals to complete this study due to rederiving their colony. After consultation with the contract officer representative, Charles River Technical Staff and Battelle's Veterinary Staff, a wider weight range was deemed acceptable. Charles River will try to deliver guinea pigs of similar weight.

Impact on the Study:

There are no adverse affects on the study with this change. This should expedite the completion of the *in vivo* portion of this study.

Change No. 2

Change: Delete the following sentense in section 9.A.(6) Housing.

"Animals are housed individually in polycarbonate cages equipped with automatic watering systems."

Replace with the following paragraph.

"Animals are housed individually. Caging may be polycarbonate cages or stainless steel equipped with automatic watering systems during quarantine and maintenance. After HD exposure, polycarbonate cages lined with an appropriate cage liner (such as rabbit pan liners cut to fit) will be used in the fume hoods."

Reasons for Change:

There is concern that bedding may damage or irritate dosing sites and this can be minimized by maintaining animals singlely and in stainless steel cages. Room temperature is elevated to reduce cold stress and animals are maintained in the stainless steel caging for less than two weeks (generally one week).

Impact on the Study:

This change will not affect the integrity of the study. No adverse health effects nor changes in experimental parameters have been observed.

Change No. 3

Change: Delete the following sentense in section 9.A.(8) Temperature.

"Maintained at 60 F ($\pm$ 5 F)"

Replace with the following paragraph.

"Maintained between 65 to 75 F. At least 90 percent of the total twice-daily measurements will fall within this range."

Reasons for Change:

The temperature range was recommended by the producer of the hairless guinea pig.

Impact on the Study:

This change will not affect the integrity of the study. No adverse health effects nor changes in experimental parameters have been observed.

Change No. 4

Change: Delete the following sentense in section 9.A.(9) Humidity.

"Maintained at 50 percent (± 10 percent)."

Replace with the following paragraph.

"Maintained between 40 to 60 percent humidity. At least 90 percent of the total twice-daily measurements will fall within this range."

Reason for Change:

The humidity range was recommended by the producer of the hairless guinea pig.

Impact of Change:

This change will not affect the integrity of the study. No adverse health effects nor changes in experimental parameters have been observed.

Change No. 5

Change: Delete the following statement in section 9.A.(10) Diet.

"Purina Certified Rodent Chow pellets ...."

Replace with the following statement.

"Purina Guinea Pig Chow or equivalent ..."

Reason for Change:

The protocol referred to rodent chow and not guinea pig chow.

Impact of Change:

This change will not affect the integrity of the study. No adverse health effects nor changes in experimental parameters have been observed since guinea pig chow is being fed.

Change No. 6

Change: Delete the following paragraph in section 10.A.(2) Anesthetization.

"Guinea pigs are anesthetized by intramuscular (i.m.) injection of ketamine (30 mg/kg) and xylazine (6 mg/kg). Booster injections of ketamine (30 mg/kg) and xylazine (6 mg/kg) will be given as needed."

Replace with the following statement.

"Guinea pigs are anesthetized by intramuscular (i.m.) injection of an appropriate anesthetic, such as ketamine and xylazine. Booster injections of ketamine and xylazine will be given as needed. Dosages are provided by the veterinary staff."

Reason for Change:

The dosage stated does not appropriately anesthetize the more recently derived hairless guinea pig. A change in the dosage and possibly anthesthetic may be needed.

Impact of Change:

This change will not affect the integrity of the study. No adverse health effects nor changes in experimental parameters have been observed.

Approved by:

James A. Blank, Ph.D., D.A.B.T.
Study Director

12/22/95
Date

LTC Richard R. Stotts, D.V.M., Ph.D.
USAMRICD COR

4 JAN 96
Date

A Comparison of the Biochemical Changes Produced by Vesicating and Non-vesicating Alkylating Agents in Normal Human Epidermal Keratinocytes

Protocol Ammendment 4

Change 1: Page 5, Section 10.A.3. Marking of Test Sites, first paragraph.

Add the following sentence to the end of the paragraph:

A seventh application site/dosing grid may be added to the six site grid for a vehicle control group.

Reason for Change:

An additional vehicle control for histopathology is needed. Another grid site was added to the grid of six dosing sites to evaluate the vehicle.

Impact of Change:

This change will not adversely affect the integrity of the study. Addition of the vehicle control site to the study allows the researcher to evaluate the effect of the vehicle control on the endpoints being measured.

Change 2: Page 6, Section 10.A.3. Marking of Test Sites, second paragraph.

Delete the following paragraph:

Each dose site will contain either a different alkylating agent or a different dose of the same alkylating agent. The "E" site will contain the negative control (no dose) and the "F" site will contain the positive control (HD dose).

Add to the end of the following sentence:

Each dose site will contain either a different alkylating agent, a different dose of the same alkylating agent, or an appropriate solvent/vehicle. The dosing sites may be abraded, if the ability of the alkylating agent and/or its vehicle to penetrate the strata corneum is questioned. The "E" site will contain the negative control (no dose), the "F" site will contain the positive control (HD dose), and the "G" site will contain the vehicle control site.

Reason for Change:

Review of the current protocol and initial results raises the question whether compounds are able to penetrate the strata corneum. If an alkylator cannot penetrate, is the lack of pathology related to penetration problem or lack of vesicating effect? Abrading the strata corneum should eliminate lack of penetration as a variable. Abraded dose sites for histopathology is needed. To evaluate the vehicle and abraded skin, another grid site was added to the six grid dosing sites.

Impact of Change:

This change will not adversely affect the integrity of the study. Addition of a vehicle control site and abrading some of the dosing sites, where penetration of the alkylating agent is in question, allows the researcher to evaluate the effect of these parameters on the enpoints being measured.

Approved by:

James A. Blank, Ph.D., D.A.B.T.
Study Director

3/4/96
Date

LTC Richard R. Stotts, D.V.M., Ph.D.
USAMRICD COR

4 MAR 96
Date

APPENDIX B

CHEMICAL SOLUBILITY AND STABILITY DATA

SOLUBILITY AND STABILITY ANALYSIS OF MELPHALAN, CISPLATIN, CHLOROETHYL ETHYL SULFIDE, NITROGEN MUSTARD, AND THIO-TEPA

Solubility

It was necessary to solubilize the test substances in biologically compatible solvents at concentrations near 200 mg/mL. Solubilities of the test compounds in the candidate vehicles were determined by adding 50 μL aliquots of the vehicle, to 50 mg of the test compound in a 2 mL septum sealed vial with mixing until solubility was achieved. The mixture was vortexed for approximately 30 seconds and allowed to stand for five minutes before observation. Test compounds were determined to be soluble when visual observation indicated a homogeneous mixture. The results of the solubility measurements for each test compound are summarized below.

Stability

The stability of each test compound in the vehicle of choice at -70° C was accomplished using the following procedure. A 1 mL sample of each stability solution was prepared at approximately 200 mg/mL in a 2 mL septum sealed vial. The solution was divided into 10 μL aliquots and stored in 2 mL GC vials at -70° C pending analysis. A single vial was removed at the prescribed interval and diluted to a concentration below 10 mg/mL for analysis. Standards were prepared daily with concentrations which encompassed the expected concentration of the test substance. Sample analysis methods and stability profiles for each test compound are summarized below.

1. *Nitrogen Mustard*

   Nitrogen mustard was found to be soluble in RPMI at a concentration of 200 mg/mL. Nitrogen mustard/RPMI stability aliquots were removed at the prescribed interval and diluted with ethanol, which contained 2 mg/mL of n-nonol as an internal standard. The samples were analyzed by gas chromatography for a period of 19 days. Gas chromatographic conditions and stability profiles for nitrogen mustard are given in Attachment A. Nitrogen mustard did not show significant decomposition ($<$ 4 percent) during the course of the study.

2. *Chloroethyl Ethyl Sulfide (CEES)*

   CEES was found to be soluble in RPMI at a concentration of 200 mg/mL. CEES/RPMI stability aliquots were removed at the prescribed interval and extracted, by vortexing for 30 seconds with 1 mL of methylene chloride which contained 2 mg/mL of butyl sulfide as an internal standard. The samples were analyzed by gas chromatography for a period of 26 days. Gas chromatographic conditions and stability profiles for CEES are given in Attachment B. CEES did

not show significant decomposition (< 6 percent) during the course of the study.

3. *Melphalan*

Melphalan was found to be soluble in ethanol at a concentration of 200 mg/mL. Melphalan/ethanol stability aliquots were removed at the prescribed interval and diluted with ethanol, which contained 2 mg/mL of propyl 4-hydroxybenzoate as an internal standard. The samples were analyzed by high performance liquid chromatography for a period of 11 days. Liquid chromatographic conditions and stability profiles for melphalan are given in Attachment C. Melphalan did not show significant decomposition (< 6 percent) during the course of the study.

4. *Cisplatin*

Cisplatin did not exhibit significant solubility in any of the candidate solvents including RPMI, saline, ethanol, multisol, PEG 200, or pentane. However cisplatin did form a suspension in PEG 200 and multisol at concentrations of 200 mg/ml. No acceptable methodology for analysis of cisplatin has been established.

5. *Thio-TEPA*

Thio-TEPA was found to be soluble in ethanol at a concentration of 200 mg/mL. No acceptable methodology for analysis of thiotepa has been established.

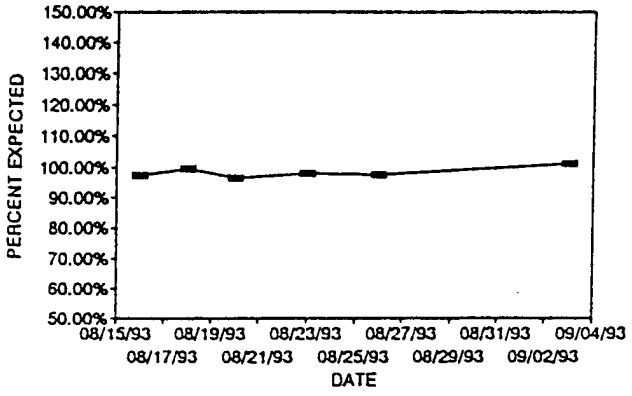
ATTACHMENT A
Nitrogen Mustard

CONTROL CHART
NITROGEN MUSTARD STABILITY ANALYSIS

ANALYST: SCOTT CHAFFINS
INSTRUMENT: HP 5890 ,A/D 0
COLUMN: HP-5, 25M x 0.32mm I.D. x .3um film thickness
FLOW RATES: Carrier 30 cm/sec. He, Carrier & Make-up 30 ml/min.
Hydrogen 35 ml/min., Air 350 ml/min.
OVEN PROGRAM:50(0)-250(1)@20C/min. Post Temp. 290C(0)
TEMP. ZONES: DETECTOR 275C, INJECTOR 250C
SAMPLE SIZE: 1 ul SPLIT(PURGE FLOW=30mL/min)
DATA SYSTEM: HP3350/LAS (A/D COLLECTION 16Hz)ATTN=2^0
SPREADSHEET: QUATTRO PRO (Release 4.0)

SAMPLE IDENTIFICATION	ANALYSIS DATE	SAMPLE RECOVERY	RECOVERY VS. DAY 0	STATISTICS	
N-MUSTARD 2.0 MG/ML	08/16/93	97.35%	100.00%	AVG=	98.33%
N-MUSTARD 2.0 MG/ML	08/18/93	99.51%	102.22%	STD=	0.02
N-MUSTARD 2.0 MG/ML	08/20/93	96.46%	99.09%	RSD=	1.73%
N-MUSTARD 2.0 MG/ML	08/23/93	97.95%	100.62%		
N-MUSTARD 2.0 MG/ML	08/26/93	97.57%	100.23%		
N-MUSTARD 2.0 MG/ML	09/03/93	101.13%	103.88%		

STABILITY ANALYSIS PLOT
NITROGEN MUSTARD/RPMI



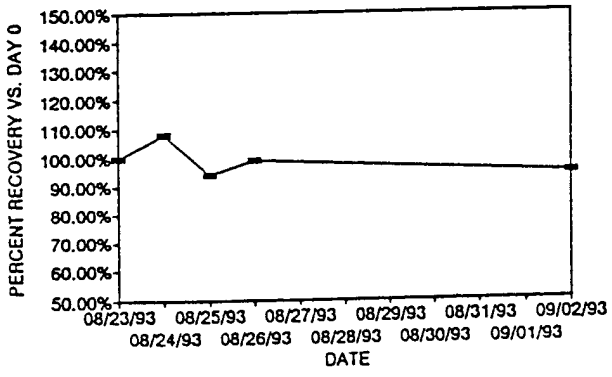
ATTACHMENT B
Chloroethyl Ethyl Sulfide

SUMMARY ANALYSIS REPORT
MELPHALAN STABILITY ANALYSIS

ANALYST: SCOTT CHAFFINS
ANALYSIS METHOD: DIODE ARRAY HPLC
INSTRUMENTATION: WATERS ULTRAWISP 715, WATERS 600E PUMP,
WATERS 991 DIODE ARRAY DETECTOR AT 269nm
COLUMN: SUPELCOSIL LC-1 25 cm x 4.6 mm, 5 MICRON
MOBILE PHASE: 50% BUFFER(1.0 mL ACETIC ACID, 1 mL TRIETHYLAMINE IN 1000 mL H2O)
50 % METHANOL
FLOW RATE: 1.5 ml/min
COLUMN TEMP.: 35 C
SAMPLE SIZE: 60 ul
DATA SYSTEM: HP3350/LAS (A/D COLLECTION 8 Hz) ATTN=2^0
SPREADSHEET: QUATTRO PRO VERSION 4.0

SAMPLE IDENTIFICATION	ANALYSIS DATE	SAMPLE RECOVERY	RECOVERY VS. DAY 0	STATISTICS	
MELPHALAN 2.0 mg/mL	08/23/93	101.07%	100.00%	AVG=	100.07%
MELPHALAN 2.0 mg/mL	08/24/93	109.16%	108.00%	STD=	0.06
MELPHALAN 2.0 mg/mL	08/25/93	94.94%	93.93%	RSD=	5.80%
MELPHALAN 2.0 mg/mL	08/26/93	100.12%	99.06%		
MELPHALAN 2.0 mg/mL	09/02/93	95.07%	94.06%		

STABILITY ANALYSIS PLOT
MELPHALAN/ETHANOL



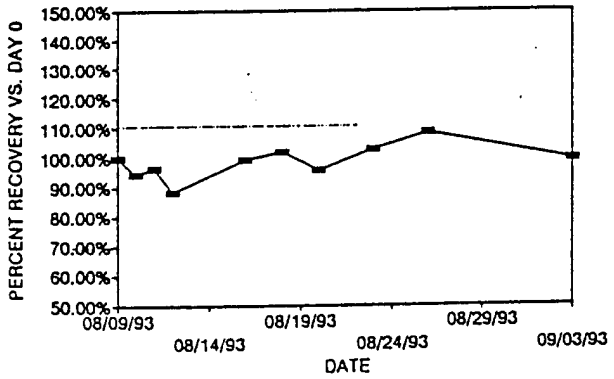
ATTACHMENT C
Melphalan

CONTROL CHART
CHLOROETHYL ETHYL SULFIDE STABILITY ANALYSIS

ANALYST: SCOTT CHAFFINS
INSTRUMENT: HP 5890 ,A/D 1
COLUMN: HP-5, 25M x 0.32mm I.D. x .3um film thickness
FLOW RATES: Carrier 30 cm/sec. He, Carrier & Make-up 30 ml/min.
Hydrogen 35 ml/min., Air 350 ml/min.
OVEN PROGRAM:50(0)-250(1)@20C/min. Post Temp. 290C(0)
TEMP. ZONES: DETECTOR 275C, INJECTOR 25JC
SAMPLE SIZE: 1 ul SPLIT(PURGE FLOW=30mL/min)
DATA SYSTEM: HP3350/LAS (A/D COLLECTION 16Hz)ATTN=2^0
SPREADSHEET: QUATTRO PRO (Release 4.0)

SAMPLE IDENTIFICATION	ANALYSIS DATE	SAMPLE RECOVERY	RECOVERY VS. DAY 0	STATISTICS	
CEES 1.0 MG/ML	08/09/93	35.00%	100.00%	AVG=	98.57%
CEES 1.0 MG/ML	08/10/93	33.06%	94.46%	STD=	0.05
CEES 1.0 MG/ML	08/11/93	33.66%	96.17%	RSD=	5.48%
CEES 1.0 MG/ML	08/12/93	30.88%	88.23%		
CEES 1.0 MG/ML	08/16/93	34.72%	99.20%		
CEES 1.0 MG/ML	08/18/93	35.60%	101.71%		
CEES 1.0 MG/ML	08/20/93	33.54%	95.83%		
CEES 1.0 MG/ML	08/23/93	35.93%	102.66%		
CEES 1.0 MG/ML	08/26/93	37.93%	108.37%		
CEES 1.0 MG/ML	09/03/93	34.68%	99.09%		

STABILITY ANALYSIS PLOT
CHLOROETHYLETHYL SULFIDE/RPMI



APPENDIX C

SCORES FOR ANIMALS IN PRELIMINARY HD DOSE AND EXPERIMENTAL PROCEDURE EVALUATION

TABLE C-1. SCORES FOR ANIMALS IN PRELIMINARY HD DOSE AND EXPERIMENTAL PROCEDURE EVALUATION

HD Dose (μL)	Animal Number	Epidermal Necrosis	Follicular Necrosis	Microblister Formation	Intracellular Edema	Pustular Epidermitis
Vehicle Control	1	1	0	0	0	0
	2	1	0	0	0	0
	3	0	0	0	0	1
	4	0	0	0	0	0
	5	1	0	0	0	0
	6	1	0	0	0	0
	7	1	0	0	0	0
	8	1	0	0	0	0
	MEAN	0.8	0.0	0.0	0.0	0.1
	STD	0.5	0.0	0.0	0.0	0.4
0.25	1	4	4	2	1	1
	2	3	4	3	2	1
	3	4	2	4	1	3
	4	4	4	3	1	0
	5	4	4	4	1	2
	6	4	3	3	1	1
	7	4	4	3	1	1
	8	4	4	4	1	3
	MEAN	3.9	3.6	3.3	1.1	1.5
	STD	0.4	0.7	0.7	0.4	1.1
0.50	1	2	4	2	2	0
	2	4	4	4	1	1
	3	3	4	3	1	1
	4	4	4	3	1	0
	5	4	3	4	1	1
	6	4	4	3	0	3
	7	4	4	3	1	2
	8	4	4	3	1	3
	MEAN	3.6	3.9	3.1	1.0	1.4
	STD	0.7	0.4	0.6	0.5	1.2
1.0	1	3	4	1	1	0
	2	3	4	2	1	1
	3	4	4	2	1	2
	4	4	3	2	1	2
	5	4	4	3	1	3
	6	2	4	1	1	1
	7	3	4	2	1	0
	8	3	4	2	1	0
	MEAN	3.3	3.9	1.9	1.0	1.1
	STD	0.7	0.4	0.6	0.0	1.1

APPENDIX D

INITIAL LETTER REPORT ON TASK 91-23
DATED SEPTEMBER 14, 1993

For Review and Approval

Name		Initials	Date
Originator	JA Blank		9/14/93
Concurrence	DW Korte		14 Sept 93
Approved	DW Hobson		9/15/93

No. G1555-9001 (846)

Internal Distribution

JA Blank
TH Snider
RMO
DW Hobson/File

September 14, 1993

LTC Don W. Korte, Jr., MS
Battelle Columbus Operations
505 King Avenue
Building JM-3
Columbus, OH 43201-2693

Dear LTC Korte:

Contract No. DAMD17-89-C-9050
Letter Report on Task 92-23

Introduction

This interim letter report presents data from studies performed at the Medical Research and Evaluation Facility (MREF) under Task 92-23, "A Comparison of the Biochemical Changes Produced by Vesicating and Non-Vesicating Alkylating Agents in Normal Human Epidermal Keratinocytes" to assess microblister formation in the hairless guinea pig (HGP) model following percutaneous sulfur mustard (HD) exposure. In this task, preliminary in vivo studies are performed prior to in vitro comparative studies to confirm the vesicating potential of selected alkylating agents. HD which is known to produce microvesication in the HGP was examined first to establish liquid HD exposure conditions. Liquid HD would then be used as a positive control during the evaluation of the remaining alkylating agents. This letter report summarizes data concerning establishment of a liquid HD exposure as the positive in vivo assay control for microvesication. These studies were performed in accordance with MREF Protocol 87 (Attachment A).

Materials and Methods

HD, along with data regarding its identity and purity, was obtained from the U.S. Army Medical Research Institute for Chemical Defense (USAMRICD). Male euthymic, HGPs were obtained from Charles River Laboratories.

Animal Preparation - The day prior to the study, the animals were weighed and eight animals weighing between 250 and 350 gm were selected for study. The backs of the HGPs were gently wiped with isopropanol. On the day of the study, the animals were anesthetized by intramuscular (i.m.) injection of ketamine (35 mg/kg) and xylazine (6 mg/kg) and placed in sternal recumbency with all legs taped to a tie-down board. A dosing grid, as shown below, was applied to the dorsum of each animal using a felt-tipped pen.

Anterior <——————————> Posterior

	A	C	E	Right
Midline				
	B	D	F	Left

The dosing sites for each animal were completely randomized using a block design. Each of the six dose sites measured approximately 2 by 2 cm. Four of the dose sites were either not dosed or dosed with 0.25, 0.50, or 1 μL of neat HD. The two remaining dose sites were used to establish the effectiveness of the decontamination process and hence the safety of submitting samples for histological evaluation. These latter two sites were later extracted with organic solvent and the level of extractable HD determined.

HD Exposure - HD was applied as a 1 cm streak. The dose levels of HD were either 0.25, 0.50, or 1 μL of neat HD. Two hours after dosing, the dose sites including the non-exposed control sites were decontaminated by applying a 0.5 percent sodium hypochlorite solution gently using a cotton-tipped applicator and then washing with water. Elizabethan collars, made of cardboard, were placed on the animals. The animals were housed within the fume hood in individual polycarbonate cages equipped with watering bottles for the remainder of the 24-hour exposure period.

Biopsy of Dosed Sites - At the end of the 24-hr exposure period, the animals were euthanized by pneumothorax performed under halothane anesthesia. The entire dorsum was excised using a sharp pair of scissors, then each dose site was sectioned with a sharp pair of scissors. The sections were placed into a specimen cassette which had been labeled with animal number and dose site letter, study number, protocol number, and Principal Investigator. The samples were immediately submerged in 10 percent neutral buffered formalin. After 24 hr, the two specimens exposed for HD chemical determination purposes were removed and the amount of extractable HD was determined analytically. As no HD could be detected in the tissue extract or in the formalin, it was considered safe to submit the samples for histopathology. The specimens were submitted for histopathological evaluation on the fifth day after being submerged in the neutral buffered formalin solution.

Histopathological Evaluation - The samples were trimmed in an anterior-to-posterior orientation, through the thickest portion of the lesion, if grossly discernable. Adjacent normal skin was left attached to each sample for comparison. Trimmed samples were routinely processed for paraffin embedding using alcohol and xylene in a Miles Scientific VIP 2000/3000 automatic tissue processor. The paraffin-embedded tissues were cut in 5 micron thick sections, stained with hematoxylin and eosin, and examined microscopically. Lesions were evaluated for the presence/absence of various histopathological markers:

- Intracellular Edema (ballooning degeneration, hydropic degeneration, vacuolar degeneration) of the Epidermis - Characterized by increased size, cytoplasmic pallor, and displacement of the nucleus to the periphery of affected cells; refers to all layers of the epidermis.

- Epidermal Necrosis - Primarily refers to the nuclear morphology of those cells in the epidermis; includes condensation and shrinkage (pyknosis), fragmentation (karyorrhexis), and dissolution (karyolysis) of the nucleus. Basal cells are the cells most affected by HD.

- Pustular Epidermitis - The presence of neutrophils within the epidermal layer under normal conditions and without the appropriate stimuli inflammatory mediator release; there should be no neutrophils present.

- Microblister - A visible separation and loss of attachment (at the light microscope level, i.e., 400x) of the basal cell layer from the underlying structures (i.e., basement membrane; not visible with routine staining). At a minimum, a microblister must represent the loss, or dissolution, of two adjacent basal cells. Frequently within this newly created space there will be cellular debris, neutrophils, and/or macrophages.

- Follicular Necrosis - Refers to the necrosis of the basal cell layer and other epidermal layers which are found invaginating into the dermis and lining the hair follicles.

The severity of the markers was graded from one to four, with four being the most severe. The specimens were evaluated without knowledge of the actual HD exposure levels.

Results

In all guinea pigs, the control site had negligible pathology. The skin was essentially normal except for isolated necrotic basal cells which occurred in six of the eight animals. In the remaining sites, the affected epidermis was uniformly and diffusely killed, having the

appearance of a heat or chemical burn. In these affected sites, the epidermis varied in appearance only slightly, due mainly to the degree of pyknosis of basal or other epidermal cells, the degree of neutrophilic infiltration and the degree of separation of basal cells from the underlying dermis. The scores for these evaluations are provided in Attachment B. Representative photographs of each lesion are provided in Attachment C. A summary of the histopathological observations are provided below:

		HD Dose (μL)			
		0	0.25	0.5	1
Intracellular Edema	Mean	0.0	1.1	1.0	1.0
	STD	0.0	0.4	0.5	0.0
Epidermal Necrosis	Mean	0.8	3.9	3.6	3.3
	STD	0.5	0.4	0.7	0.7
Pustular Epidermitis	Mean	0.1	1.5	1.4	1.1
	STD	0.4	1.1	1.2	1.1
Microblister	Mean	0.0	3.3	3.1	1.9
	STD	0.0	0.7	0.6	0.6
Follicular Necrosis	Mean	0.0	3.6	3.9	3.9
	STD	0.0	0.7	0.4	0.4

Discussion/Recommendation

Percutaneous liquid HD exposures produced similar effects across the dose levels examined. In all treated sites, there was extensive epidermal and follicular necrosis in all lesions regardless of dose. Microblisters were also observed in all lesions. Microblisters were observed anywhere along the site where the epidermis was necrotic. There did not appear to be a difference between microblister formation in the center versus edge of the lesion. One difference observed between dose groups was fewer microblisters occurring in the high HD

dose group. This could possibly have occurred due to rapid toxicity to the epidermal and dermal layers, including capillary and resident leukocyte toxicity which could have blunted an inflammatory cell response with edema and microblister formation.

The purpose of these studies was to identify an HD dose and liquid exposure procedure that would produce reproducible microvesication in the HGP model. This HD dose would serve as a positive control in subsequent studies to be performed under Task 91-23 to confirm the vesicating potential or lack thereof for 5 other alkylating agents. The results of these studies indicate that liquid HD, when applied as a 1 cm streak, can consistently produce microvesication at all dose levels. From these studies, a 0.25 μL HD dose is recommended for use as a positive control in subsequent studies under Task 91-23.

If you have any further questions, please feel free to contact me at (614) 424-5024 or Dr. David Hobson at (614) 424-5024.

Sincerely,

James A. Blank

James A. Blank, Ph.D.
Principal Research Scientist

JAB/tsk

Attachments

cc: COL Charles G. Hurst, MC, Commander, USAMRICD
LTC James S. Little, MS, Deputy Commander, USAMRICD
LTC David H. Moore, VC, RAD V, USAMRDC
LTC Robert B. Moeller, Pathophysiology Division, USAMRICD
LTC James A. Romano, MS, TAM, USAMRICD
Dr. David E. Lenz, Pharmacology Division, USAMRICD (2)
Ms. Ellen K. Mackenzie, Chief, PCMB, USAMRICD

ATTACHMENT A

MREF Protocol 87

A Comparison of the Biochemical Changes Produced by Vesicating and Non-Vesicating Alkylating Agents in Normal Human Epidermal Keratinocytes

Study performed by Battelle Columbus Operations,
505 King Avenue, Columbus, OH 43201-2693

1. Principal Investigator and Manager: David W. Hobson, Ph.D., D.A.B.T., Medical Research And Evaluation Facility (MREF)

2. Study Director: James A. Blank, Ph.D.

3. Study Veterinarian: Allan G. Manus, D.V.M.

4. Sponsor: U.S. Army Medical Research and Development Command (USAMRDC)

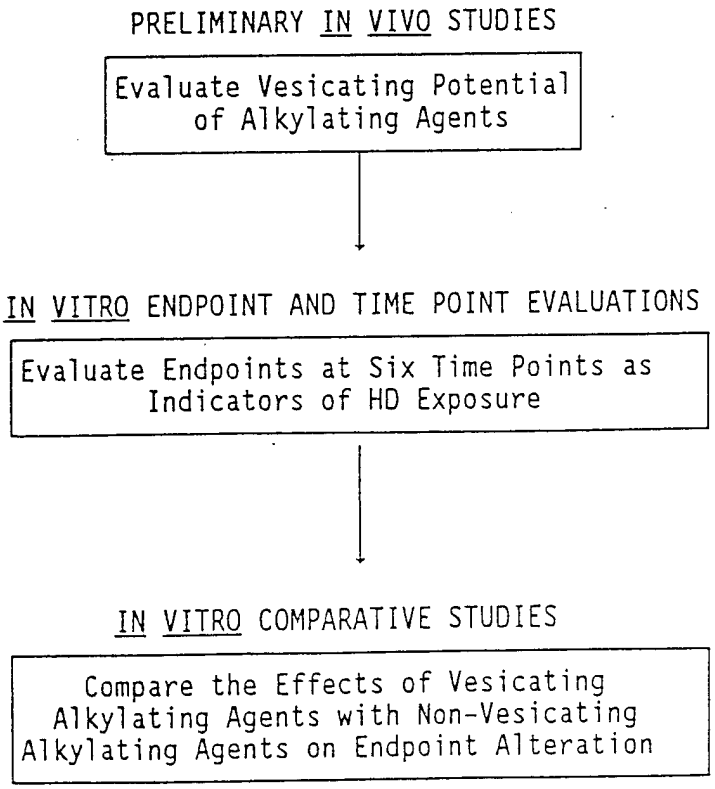
5. Sponsor Monitor: LTC Don W. Korte, Jr., U.S. Army Medical Research Institute of Chemical Defense (USAMRICD)

6. Background: The USAMRDC is performing research to characterize the pathophysiology produced by sulfur mustard (HD), in order to design effective therapeutic interventions. There are a number of biochemical parameters, such as poly(ADP-ribose) polymerase (PADPRP) activation, nicotinamide adenine dinucleotide (NAD^+) depletion, adenosine triphosphate (ATP) depletion, decreased glucose utilization, decreased lactate production, and protease secretion, which have been associated with sulfur mustard (HD)-induced cellular toxicity. HD is a bifunctional alkylating agent which possesses vesicating properties. Although HD, like many other alkylating agents, is thought to exert its toxic effects primarily through irreversible binding and damaging of the deoxyribonucleic acid (DNA) of cells, the mechanism of HD-induced vesication is unknown. As not all alkylating agents are vesicants, a comparison of biochemical events altered by HD with the effect of other vesicating and nonvesicating alkylating agents on these same endpoints, may provide insight into the biochemical mechanism(s) involved in the vesicating process. The identification of such cellular events may provide new directions for developing therapeutic regimens for percutaneous HD exposure.

7. Objective: The objective of studies conducted under this protocol for MREF Task 91-23, "A Comparison of the Biochemical Changes Produced by Vesicating and Non-Vesicating Alkylating Agents in Normal Human Epidermal Keratinocytes", is to examine various biochemical endpoints *in vitro* using human epidermal keratinocytes (HEK) exposed to either non-vesicating (e.g., cis-platinum, melphalan, thiotepa) or vesicating (e.g., HD, chloroethyl sulfide, mechlorethamine) alkylating agents in order to provide a comparative dataset from which biochemical endpoints uniquely associated with tissue vesication may be identified.

8. Experimental Overview: For these studies, three non-vesicating alkylating agents, and three vesicating alkylating agents will be used for the comparative studies. To confirm the vesicating potential of the alkylating agents selected for use in the in vitro comparative tests, preliminary in vivo studies examining microblister formation in hairless guinea pigs will be performed. Final selection of the alkylating agents for the comparative in vitro studies will be made in conjunction with the USAMRICD Technical Point of Contact (POC), Task Area Manager (TAM), and Contracting Officer's Representative (COR), following completion of the preliminary in vivo tests.

Preliminary in vitro studies with HD will be performed to identify endpoints which respond strongly to HD exposure and to establish the time points at which these responses occur. These preliminary studies will provide an HD dataset for inclusion in the comparison of the alkylating agents and will serve to minimize the number of endpoints and time points used for subsequent comparative in vitro studies involving the other five alkylating agents. In vitro comparative studies will then be performed with two vesicating alkylating agents and three non-vesicating alkylating agents to examine for differences in endpoint responses between the two types of alkylating agents. The endpoints and time points used for the in vitro comparative studies will be selected in conjunction with the POC, TAM, and COR. A flowchart for the in vivo and in vitro studies is as follows:



9. Experimental Design:

A. Animal Test System

(1) Animals - Male euthymic, hairless guinea pigs supplied by Charles River Laboratories weighing between 250 to 350 gm will be used for this study. Hairless guinea pigs were chosen because microvesication or epidermal-dermal separation has been shown to occur in these animals following percutaneous HD exposure.

(2) Initial Weight - Guinea pigs used for these studies will weigh between 250 and 350 gm.

(3) Quarantine - Animals are quarantined for a minimum 7-day period. Guinea pigs in good physical condition are then weighed and randomized into weight-homogenized treatment groups.

(4) Acclimation - If animals are not quarantined at the MREF, then all animals will be held at the MREF for at least 24 hr prior to study initiation.

(5) Animal Identification - All animals are ear tagged to retain positive identification during animal handling and observation.

(6) Housing - Animals are housed individually in polycarbonate cages equipped with automatic watering systems.

(7) Lighting - Fluorescent lighting, with a light/dark cycle of 12 hr each per day.

(8) Temperature - Maintained at 60 F (± 5 F).

(9) Humidity - Maintained at 50 percent (± 10 percent).

(10) Diet - Purina Certified Rodent Chow pellets are available as described in Battelle SOP MREF VII-003. No contaminants are known to be present in the feed that would interfere with the results of the study.

(11) Water Supply - Water is supplied from the public water system, and given ad libitum during quarantine and holding. No contaminants are known to be present in the water that would affect the results of the study.

(12) Animal Care During Test - All animals are positioned on tie-down boards for compound exposures and are retained in polycarbonate cages within the hood system during the treatment and observation

periods. No food is provided during the treatment and observations periods, but water is provided ad libitum.

(13) Laboratory Animal Welfare Practices - Battelle's Animal Resources Facilities have been registered with the U.S. Department of Agriculture (USDA) as a Research Facility (Number 31-21) since 14 August 1967 and are periodically inspected in accordance with the provisions of the Federal Animal Welfare Act. In addition, animals for use in research are obtained only from laboratory animals suppliers duly licensed by the USDA. Battelle's statement of assurances regarding the Department of Health and Human Services (DHHS) policy on humane care of laboratory animals was accepted by the Office of Protection from Research Risks, National Institute of Health (NIH) on 27 August 1973. Animals at Battelle are cared for in accordance with the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" (DHHS Publication No. NIH 78-23), and/or in the regulations and standards as promulgated by the Agricultural Research Service, USDA, pursuant to the Laboratory Animal Welfare Act of 24 August 1966, as amended (P.L. 91-579). Protocols of all experiments using animals are reviewed and approved by Battelle's Institutional Animal Care and Use Committee (IACUC) prior to initiation of the study. The program manager accepts responsibility for the proper care and use of animals in the conduct of the research describe in this protocol.

(14) Accreditation - On 31 January 1978, Battelle's Columbus Division received full accreditation of its animal-care program and facilities from the American Association for Accreditation of Laboratory Animal Care (AALAC). Battelle's full accreditation status has been renewed after every inspection since the original accreditation. The MREF is a part of the facilities granted full accreditation.

B. Cellular Test System

(1) Normal human epidermal keratinocytes (HEKs) will be the target cell type used for these studies. These cells are of human origin and are one cell type encountering HD during percutaneous exposure.

(2) Tertiary passaged HEK cultures which had been seeded at 40,000 to 50,000 cells per square cm of growth area and are approximately 75 percent confluent will be used for these studies. The cells are received as primary cryopreserved HEKs and will be expanded as secondary cultures in tissue culture flasks (T-flask; 6 - 30 square cm flask per HEK cryopreserved ampule) prior to being passaged as tertiary cultures in microtest plates.

(3) HEKs cultures will be maintained at 37 C (± 0.5 C), 5 percent carbon dioxide (± 0.5 percent) in air mixture, and a saturated relative humidity atmosphere.

C. Test Material - Chemical surety materiel (CSM) will be supplied by USAMRICD. Purity, appropriate identification (batch number, lot number, state), stability data, and material safety data sheets will be supplied by USAMRICD.

(1) Purity and stability are confirmed periodically by the MREF for CSM stored at the MREF.

(2) Surety, security, and safety procedures for the use of CSM are thoroughly outlined in facility plans, in personnel requirements for qualifications to work with CSM, and in standard operating procedures for the storage and use of CSM.

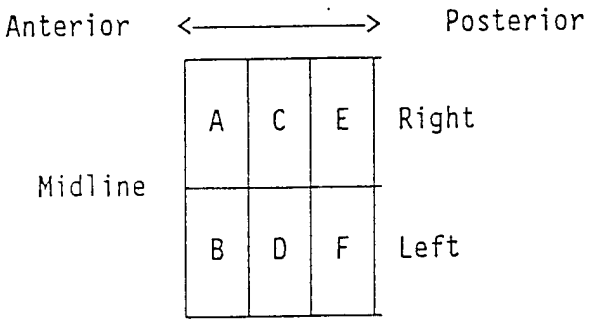
10. Preliminary In Vivo Testing - This test is performed to assess the vesicating potential of alkylating agents when percutaneously applied at 24 hr post exposure. The extent of microblister formation is determined by histopathologic examination of exposed tissue.

A. Animal Preparation for Testing -

(1) Randomization - The day prior to the study, animals are weighed and randomized into weight homogenized treatment groups. The animal backs are cleaned with ethanol or isopropanol.

(2) Anesthetization - Guinea Pigs are anesthetized by intramuscular (i.m.) injection of ketamine (30 mg/kg) and xylazine (6 mg/kg). Booster injections of ketamine (30 mg/kg) and xylazine (6 mg/kg) will be given as needed.

(3) Marking of Test Sites - Animals are anesthetized per Section 10.A.(1) then placed in sternal recumbency with all legs taped to a tie-down board. An application site/dosing grid as shown below is applied to the dorsum of each animal using a felt-tipped pen.



Each dose site will contain either a different alkylating agent or a different dose of the same alkylating agent. The "E" site will contain the negative control (no dose) and the "F" site will contain the positive control (HD dose).

B. Alkylator Agent Exposures - Compounds to be evaluated for vesication will be maximally solubilized in an appropriate solvent and applied as a liquid exposure. The alkylating agents are applied as a 1 cm streak and will be delivered using a Hamilton microliter syringe. The animals will be kept in individual polycarbonate cages equipped with watering bottles contained within the fume hood for the remainder of the 24-hour exposure period.

C. Biopsy of Dosed Sites - At the end of the 24 hr exposure period, the animals are euthanitized by over exposure to halothane. Halothane saturated gauze is placed in the bottom of a bell jar, the halothane is allowed to vaporize, then the animals are placed in the jar. The skin sections are excised and placed in a container of 5 percent neutral buffered formalin. .The samples must sit in the neutral buffered formalin solution for 24 hr before being submitted for histopathologic examination.

11. HD Evaluation Studies - Preliminary in vitro studies will be performed with HD to identify cellular and biochemical endpoints which respond most strongly to HD challenge and to aid in selecting the time points at which these endpoints will be examined in the subsequent in vitro comparative studies. These studies consist of an HD concentration response to establish the relationship between HD concentration and HEK viability at 24 hr post exposure and a time-course studies using fixed HD concentrations to evaluate the impact of HD on the endpoints listed in Table 1.

A. HD Concentration Response Study - A concentration response study will be performed to establish the relationship between HD concentration and cellular viability at 24 hr post exposure. Viability will be measured using a propidium iodide exclusion method as described in Section 13.A. This study will utilize eight HD concentrations and will be repeated two times to obtain good estimates of the fixed HD challenge concentrations for use in subsequent time-course studies. Each experimental group will consist of triplicate observations. From these data, the HD concentrations that decrease cellular viability by 25 percent (IC_{25}), 50 percent (IC_{50}), and 75 percent (IC_{75}) will be estimated and used as the fixed HD challenge concentrations in the time-course studies described below.

B. Time-Course Study - These studies examine the endpoints listed in Table 1 at six different times following exposure to each of the fixed HD challenge concentrations as well as a vehicle (tissue culture

medium) control. Each experimental group will consist of triplicate observations and the study will be repeated two times. The concentration and time-course studies will produce an approximate 2500 datapoints (Table 1).

TABLE 1. TECHNICAL DETAIL FOR CONCENTRATION RESPONSE AND TIME-COURSE STUDIES

Study Type	Number Alkylating Agents	Number Treatment Groups[a]	Number Time Points[b]	Number Observations Per Treatment Group	Total Number Experiments	Data Points Total
Conc. Response	1	8	1	3	3	72
Time Course:						
•Cytometry	1	3	6	3	3	162
•NAD^+	1	3	6	3	3	162
•ATP	1	3	6	3	3	162
•Glucose Utilization	1	3	6	3	3	162
•Lactate Production	1	3	6	3	3	162
•PADPRP Activity	1	3	6	3	3	162
•Prostaglandin E_2	1	3	6	3	3	162
•Leukotriene B_4	1	3	6	3	3	162
•Interleukin-1	1	3	6	3	3	162
•Chromogenic Substrate 1	1	3	6	3	3	162
•Chromogenic Substrate 2	1	3	6	3	3	162
•Chromogenic Substrate 3	1	3	6	3	3	162
•Chromogenic Substrate 4	1	3	6	3	3	162
•Chromogenic Substrate 5	1	3	6	3	3	162
•Chromogenic Substrate 6	1	3	6	3	3	162
					Total	2,502

[a]Number of treatment groups per alkylating agent in addition to the vehicle control.
[b]Number of time points at which each treatment group will be examined.

12. In Vitro Comparative Study: In vitro comparative studies will be performed to assess whether differences in biochemical endpoint alterations as a function of non-vesicating versus vesicating alkylating agent are observed. These studies will examine the effect of three non-vesicating alkylating agents and two additional vesicating alkylating agents on endpoint alterations. The endpoints and time points used in these experiments are dependent upon the studies performed in Section 11.B. The endpoints and time points will be selected in conjunction with the USAMRICD COR, TAM, and POC.

A. Concentration Response Study - A concentration response study will be performed to establish the relationship between alkylating agent concentration and cellular viability at 24 hr post exposure. This study will utilize eight alkylating agent concentrations and will be repeated two times to obtain good estimates of the fixed challenge concentrations for use in subsequent time-course studies. Each

experimental group will consist of triplicate observations. From these data, the alkylating agent IC_{25}, IC_{50}, and IC_{75} are estimated for use as the fixed challenge concentrations in the time-course experiments.

B. Time-Course Study - These experiments consist of examining the endpoints selected from the studies involving HD at three time points following exposure to each of the fixed HD challenge concentrations as well as a vehicle control. The three time points at which all endpoints will be evaluated are selected in conjunction with the USAMRICD POC and COR based upon the studies performed with HD. Each experimental group will consist of triplicate observations and the study will be repeated two times.

13. Endpoint Measurement:

A. Cell Viability - Battelle SOP MREF V-001 and MREF Method/In Vitro No. 15 will be followed for cell viability measurements as applicable. Current methods are for measuring mononuclear leukocyte cell viability and, therefore, a new MREF method or modification of an existing MREF and USAMRICD method will be required for evaluation of this adherent cell type in a 96-well plate format. The procedure developed will utilize the following procedure:

(1) Cellular viability will be measured by flow cytometer assessing propidium iodide incorporation into nonviable cells. At the designated time, the tissue culture supernatant is transferred to a second vessel and trypsin is added to the cultures to detach the adherent cells.

(2) Once the cells have detached, trypsin neutralizing solution is added to neutralize the action of the trypsin and the entire mixture is combined with the original tissue culture supernatant.

(3) The cells are pelletized, and the majority of the supernatant is aspirated. Propidium iodide is added and the tube gently mixed, incubated for at a minimum of 5 min, then analyzed using a flow cytometer.

B. NAD^+ - NAD^+ will be measured following methodology contained in Battelle SOP MREF V-003 and MREF Method/In Vitro No. 1. The method may need to be modified so as to measure ATP from the same cellular digest.

C. ATP - ATP measurements will be made using a bioluminescent assay. ATP measurements are made on the cellular extracts used for NAD^+ analyses. Current cellular digestion procedures as outlined in MREF Method/

In Vitro No. 1 may require modification so as to use the same extract to assay ATP and NAD^+.

D. PADPRP Activity - PADPRP can modify proteins by attaching the ADP-ribose moiety of NAD^+ to the protein. PADPRP activity is measured following methodology that will be developed during the course of the task by Battelle and USAMRICD investigators. Current USAMRICD procedures use radiolabeled NAD^+ and measure incorporation of label into proteins. In order to make the assay more economical, an attempt will be made to adapt this assay to a 24-well plate system and, if possible, to a 96-well microplate system.

E. Glucose Utilization and Lactate Production - The level of glucose and lactate in the tissue culture supernatant will be assessed using a Biochemical Analyzer made by YSI, Inc. This analyzer can make rapid measurements of glucose and lactate using immobilized enzyme electrode technology.

F. Protease Activity - Tissue associated protease activity will be measured using a series of different chromogenic substrates. Protease activity is measured spectrophotometrically. The commercially available chromogenic substrates examined are Chromozym TRY, TH, U, PK, TPA, and a Collagenase substrate (PZ-Pro-Leu-Gly-Pro-D-Arg). The parameters for this assay (i.e., concentration of chromogenic substrate and length of incubation) will be defined within this task.

G. IL-1, $PG\text{-}E_2$, and $LT\text{-}B_4$ - The level of these factors in the tissue culture supernatant will be assessed by enzyme immunoassay using commercially available kits.

14. Assay Controls: HD will be used as the assay positive control for the conduct of the alkylating agent time response studies. The dataset used for these purposes is very limited as it is based only upon the HD time-course studies. If the mean value of the positive assay control falls outside three standard deviations of the mean, then the data are considered suspect. The experimental procedures will be reviewed to determine the cause of the positive assay control shift. If the cause of the extreme value cannot be determined, then the influence of this experiment on the final results will be assessed before including or omitting the dataset.

15. Record Maintenance: The following records are to be maintained for MREF Task 91-23:

A. CSM accountability log and inventory,

B. Reagent preparation,

C. Decontamination and disposal records, and

D. Any other records needed to reconstruct the study and demonstrate adherence to this protocol.

16. Reports:

A. Interim letter report covering the conduct of the in vivo studies, the HD concentration and time-course studies will be prepared and submitted.

B. At the end of Task 91-23, a Draft Final Report will be prepared and submitted to USAMRICD within 60 working days of task completion. The Draft Final report includes, at a minimum, the following sections:

(1) Signature page of key study personnel and their responsibilities,

(2) Experimental design,

(3) Test material description

(4) Tabulation and statistical data analysis, and

(5) Discussion and conclusion.

The Final Report will be submitted within 30 days of receiving the Draft Final Report comments from USAMRICD.

17. Approval Signatures:

James A. Blank
James A. Blank, Ph.D.
Study Director

13 October 1992
Date

David W. Hobson
David W. Hobson, Ph.D., D.A.B.T.
MREF Principal Investigator and Manager

15 OCT 92
Date

Allan G. Manus
Allan G. Manus, D.V.M.
Chief Veterinarian

10/16/92
Date

John W. Korte
LTC Don W. Korte, Jr.
USAMRICD COR

10/14/92
Date

A Comparison of the Biochemical Changes Produced by
Vesicating and Non-Vesicating Alkylating Agents in
Normal Human Epidermal Keratinocytes

Amendment No. 1

Change: Replace Page 6, Section 10.C. with the following

C. Biopsy of Dosed Sites - Animals exposed to HD are handled in accordance with Battelle SOP MREF II-003. At the end of the 24-hr exposure period, the animals are euthanized with halothane in the fume hood. Halothane saturated gauze is placed in the bottom of a bell jar, the halothane is allowed to vaporize, then the animals are placed in the jar. Samples of skin tissue from various test sites are harvested from euthanized animals. Unaffected skin adjacent to a test site is lifted with forceps, and the test site skin is excised with scissors and placed into 5 percent neutral buffered formalin (NBF) solution.

(1) The skin section is excised in the fume hood, the appropriate label attached, then placed into a container of NBF fixative which is held at the hood face. The cap to the fixative is put in place, the container placed in a secondary and then removed from the hood face. Each specimen is identified by placing it into a labeled jar or cassette, or by stapling an identification card to one corner of the specimen. Specimens are identified by task and protocol numbers, charge account number, study director, date and time of sample collection, animal identification number, and treatment site. Where appropriate, warning labels stating that the skin samples were exposed to HD are affixed to the outside of each container and to the outside of the box used for transportation. All HD-exposed samples are retained at the MREF for 24 hrs before being transported to another facility for histologic processing. Standard sectioning with hematoxylin and eosin staining are performed on each sample.

(2) Prior to submitting tissue samples for histopathology, tissue samples which had been exposed to the maximal HD dose (1 μL) are extracted, and HD content is determined analytically to confirm decontamination during the fixative process. In addition, the NBF solution is analyzed to determine whether HD exists after the 24-hr retention period (Battelle SOP MREF III-005). Validating these analytical methods is accomplished in the following pilot study.

(a) Extraction of HD from Dorsal Skin: One anesthetized, restrained hairless guinea pig is dosed with 1 μL of HD at each of five test sites (Battelle SOP MREF II-003). A sixth site serves as a negative control. Two hours later, the six sites are decontaminated with 0.5 percent NaOCl followed by two distilled water rinses (Battelle SOP MREF II-002). The animal is

euthanized, and the dorsal skin is excised and pinned to a cutting board. The six sites are separated with a scalpel, and each is immediately placed into a scintillation vial containing 2 mL of hexane with an internal standard. The individual transferring the tissue dons a clean set of gloves. Each vial is vigorously shaken, then opened and aliquoted into three, 1-mL GC vials for chromatographic analysis for HD (Battelle SOP MREF III-002).

(b) Extraction of HD from NBF Solution (Battelle SOP MREF III-002): A volume of 5 percent NBF solution is spiked with 1 µL of HD, q.s.'d to 100 mL in a 100-mL volumetric, sealed, and inverted several times. The volumetric seal is removed and 10 mL of its contents are aliquoted into each of three 15-mL capacity culture tubes. A 1-mL volume of hexane with internal standard is added to each culture tube, which is then sealed and shaken vigorously. Three, 1-mL volumes of each tube are aliquoted into GC vials for chromatographic analysis for HD.

Reason: Histologic processing and examination are necessary to determine microvesication in hairless guinea pigs. This change describes the handling of specimens which were previously exposed to HD.

Impact: This modification does not have an impact on this study.

Amendment No. 1 Approval Signatures:

James A. Blank, Ph.D.
Study Director — 16 April 1993 Date

David L. Stitcher
CIH, Safety & Surety Officer — 16 April 1993 Date

LTC Don W. Korte, Jr.
USAMRICD COR — 20 APR 93 Date

A Comparison of the Biochemical Changes Produced by
Vesicating and Non-Vesicating Alkylating Agents in
Normal Human Epidermal Keratinocytes

Protocol Amendment No. 2

Change 1: Page 1, Section 1.

Change to: "Co-Principal Investigator and Manager: John B. Johnson, D.V.M., Medical Research and Evaluation Facility (MREF)".

Reason for change:

Since this protocol was last revised, the principal investigator and manager has changed.

Change 2: Page 1, Section 3. Study Veterinarian.

Change to: "Tracy A. Peace, D.V.M.".

Reason for change:

Since this protocol was last revised, the study veterinarian has changed.

Change 3: Page 1, Section 4. Sponsor.

Change to: "U.S. Army Medical Research and Materiel Command (USAMRMC)".

Reason for change:

Since this protocol was last revised, the name of the sponsoring organization has been changed.

Change 4: Page 1, Section 5. Sponsor Monitor.

Change to: "LTC Richard R. Stotts, D.V.M., P.h.D., U.S. Army Medical Research Institute of Chemical Defense (USAMRICD)".

Reason for change:

Since this protocol was last revised, the sponsor monitor has changed.

Approved by:

James a Blank

James A. Blank, Ph.D., D.A.B.T.
Study Director

11/8/95
Date

Richard R Stotts

LTC Richard R. Stotts, D.V.M., Ph.D.
USAMRICD COR

9 NOV 95
Date

A Comparison of the Biochemical Changes Produced by Vesicating and Non-Vesicating Alkylating Agents in Normal Human Epidermal Keratinocytes

MREF Protocol 87. Amendment No. 3

Change Number No. 1

Change: Delete the following statement in section 9.A.(2) Initial Weight at the end of the first sentence.

"250 and 350 gm."

Replace with the following range.

"Animals will weigh 250 gm at a minimum".

Reasons for Changes:

The supplier of the hairless guinea pigs, Charles River Laboratories, has had difficulty providing animals to complete this study due to rederiving their colony. After consultation with the contract officer representative, Charles River Technical Staff and Battelle's Veterinary Staff, a wider weight range was deemed acceptable. Charles River will try to deliver guinea pigs of similar weight.

Impact on the Study:

There are no adverse affects on the study with this change. This should expedite the completion of the *in vivo* portion of this study.

Change No. 2

Change: Delete the following sentense in section 9.A.(6) Housing.

"Animals are housed individually in polycarbonate cages equipped with automatic watering systems."

Replace with the following paragraph.

"Animals are housed individually. Caging may be polycarbonate cages or stainless steel equipped with automatic watering systems during quarantine and maintenance. After HD exposure, polycarbonate cages lined with an appropriate cage liner (such as rabbit pan liners cut to fit) will be used in the fume hoods."

Reasons for Change:

There is concern that bedding may damage or irritate dosing sites and this can be minimized by maintaining animals singlely and in stainless steel cages. Room temperature is elevated to reduce cold stress and animals are maintained in the stainless steel caging for less than two weeks (generally one week).

Impact on the Study:

This change will not affect the integrity of the study. No adverse health effects nor changes in experimental parameters have been observed.

Change No. 3

Change: Delete the following sentense in section 9.A.(8) Temperature.

"Maintained at 60 F ($\pm$ 5 F)"

Replace with the following paragraph.

"Maintained between 65 to 75 F. At least 90 percent of the total twice-daily measurements will fall within this range."

Reasons for Change:

The temperature range was recommended by the producer of the hairless guinea pig.

Impact on the Study:

This change will not affect the integrity of the study. No adverse health effects nor changes in experimental parameters have been observed.

Change No. 4

Change: Delete the following sentense in section 9.A.(9) Humidity.

"Maintained at 50 percent (± 10 percent)."

Replace with the following paragraph.

"Maintained between 40 to 60 percent humidity. At least 90 percent of the total twice-daily measurements will fall within this range."

Reason for Change:

The humidity range was recommended by the producer of the hairless guinea pig.

Impact of Change:

This change will not affect the integrity of the study. No adverse health effects nor changes in experimental parameters have been observed.

Change No. 5

Change: Delete the following statement in section 9.A.(10) Diet.

"Purina Certified Rodent Chow pellets ...."

Replace with the following statement.

"Purina Guinea Pig Chow or equivalent ..."

Reason for Change:

The protocol referred to rodent chow and not guinea pig chow.

Impact of Change:

This change will not affect the integrity of the study. No adverse health effects nor changes in experimental parameters have been observed since guinea pig chow is being fed.

Change No. 6

Change: Delete the following paragraph in section 10.A.(2) Anesthetization.

"Guinea pigs are anesthetized by intramuscular (i.m.) injection of ketamine (30 mg/kg) and xylazine (6 mg/kg). Booster injections of ketamine (30 mg/kg) and xylazine (6 mg/kg) will be given as needed."

Replace with the following statement.

"Guinea pigs are anesthetized by intramuscular (i.m.) injection of an appropriate anesthetic, such as ketamine and xylazine. Booster injections of ketamine and xylazine will be given as needed. Dosages are provided by the veterinary staff."

Reason for Change:

The dosage stated does not appropriately anesthetize the more recently derived hairless guinea pig. A change in the dosage and possibly anthesthetic may be needed.

Impact of Change:

This change will not affect the integrity of the study. No adverse health effects nor changes in experimental parameters have been observed.

Approved by:

James A. Blank, Ph.D., D.A.B.T.
Study Director

12/22/95
Date

LTC Richard R. Stotts, D.V.M., Ph.D.
USAMRICD COR

4 JAN 96
Date

A Comparison of the Biochemical Changes Produced by Vesicating and Non-vesicating Alkylating Agents in Normal Human Epidermal Keratinocytes

Protocol Ammendment 4

Change 1: Page 5, Section 10.A.3. Marking of Test Sites, first paragraph.

Add the following sentence to the end of the paragraph:

A seventh application site/dosing grid may be added to the six site grid for a vehicle control group.

Reason for Change:

An additional vehicle control for histopathology is needed. Another grid site was added to the grid of six dosing sites to evaluate the vehicle.

Impact of Change:

This change will not adversely affect the integrity of the study. Addition of the vehicle control site to the study allows the researcher to evaluate the effect of the vehicle control on the endpoints being measured.

Change 2: Page 6, Section 10.A.3. Marking of Test Sites, second paragraph.

Delete the following paragraph:

Each dose site will contain either a different alkylating agent or a different dose of the same alkylating agent. The "E" site will contain the negative control (no dose) and the "F" site will contain the positive control (HD dose).

Add to the end of the following sentence:

Each dose site will contain either a different alkylating agent, a different dose of the same alkylating agent, or an appropriate solvent/vehicle. The dosing sites may be abraded, if the ability of the alkylating agent and/or its vehicle to penetrate the strata corneum is questioned. The "E" site will contain the negative control (no dose), the "F" site will contain the positive control (HD dose), and the "G" site will contain the vehicle control site.

Reason for Change:

Review of the current protocol and initial results raises the question whether compounds are able to penetrate the strata corneum. If an alkylator cannot penetrate, is the lack of pathology related to penetration problem or lack of vesicating effect? Abrading the strata corneum should eliminate lack of penetration as a variable. Abraded dose sites for histopathology is needed. To evaluate the vehicle and abraded skin, another grid site was added to the six grid dosing sites.

Impact of Change:

This change will not adversely affect the integrity of the study. Addition of a vehicle control site and abrading some of the dosing sites, where penetration of the alkylating agent is in question, allows the researcher to evaluate the effect of these parameters on the enpoints being measured.

Approved by:

James A. Blank, Ph.D., D.A.B.T.
Study Director

3/4/96
Date

LTC Richard R. Stotts, D.V.M., Ph.D.
USAMRICD COR

4 MAR 96
Date

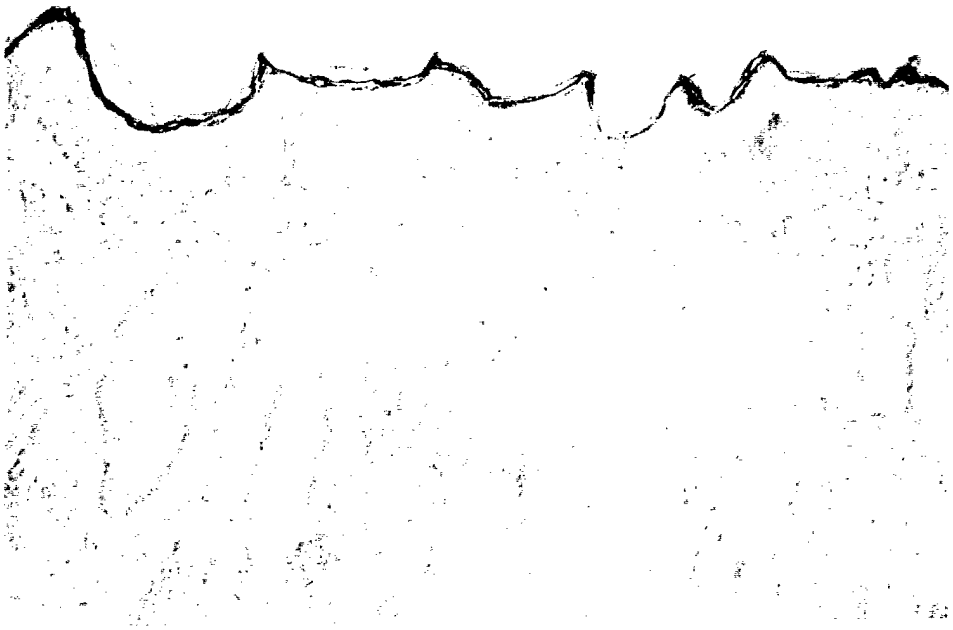
ATTACHMENT B

Scores for Evaluations

HD Dose (μL)	Animal #	Epidermal Necrosis	Follicular Necrosis	Microblister Formation	Intracellular Edema	Pustular Epidermitis
Vehicle Control	1	1	0	0	0	0
	2	1	0	0	0	0
	3	0	0	0	0	1
	4	0	0	0	0	0
	5	1	0	0	0	0
	6	1	0	0	0	0
	7	1	0	0	0	0
	8	1	0	0	0	0
	MEAN	0.8	0.0	0.0	0.0	0.1
	STD	0.5	0.0	0.0	0.0	0.4
0.25	1	4	4	2	1	1
	2	3	4	3	2	1
	3	4	2	4	1	3
	4	4	4	3	1	0
	5	4	4	4	1	2
	6	4	3	3	1	1
	7	4	4	3	1	1
	8	4	4	4	1	3
	MEAN	3.9	3.6	3.3	1.1	1.5
	STD	0.4	0.7	0.7	0.4	1.1
0.50	1	2	4	2	2	0
	2	4	4	4	1	1
	3	3	4	3	1	1
	4	4	4	3	1	0
	5	4	3	4	1	1
	6	4	4	3	0	3
	7	4	4	3	1	2
	8	4	4	3	1	3
	MEAN	3.6	3.9	3.1	1.0	1.4
	STD	0.7	0.4	0.6	0.5	1.2
1.0	1	3	4	1	1	0
	2	3	4	2	1	1
	3	4	4	2	1	2
	4	4	3	2	1	2
	5	4	4	3	1	3
	6	2	4	1	1	1
	7	3	4	2	1	0
	8	3	4	2	1	0
	MEAN	3.3	3.9	1.9	1.0	1.1
	STD	0.7	0.4	0.6	0.0	1.1

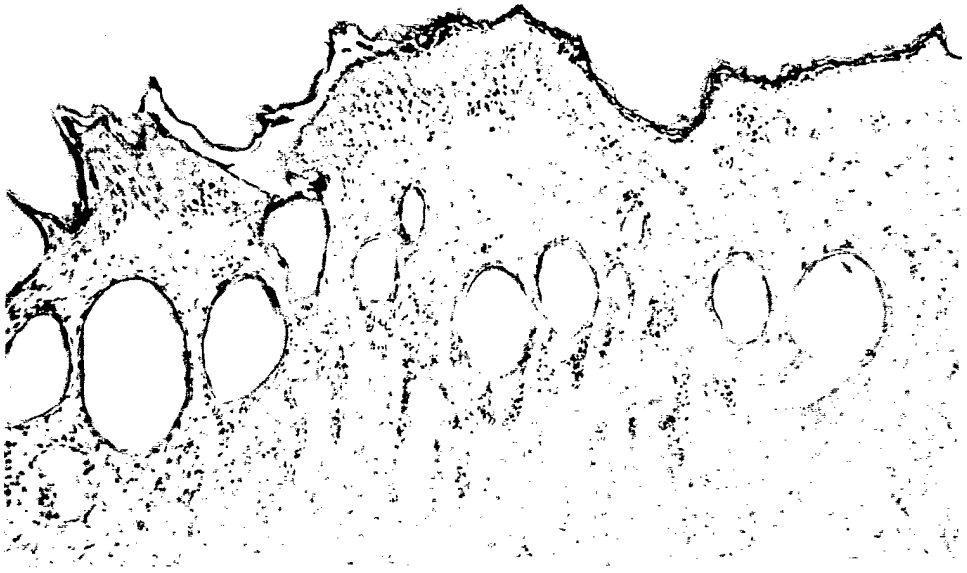
ATTACHMENT C

Photographs of Each Lesion

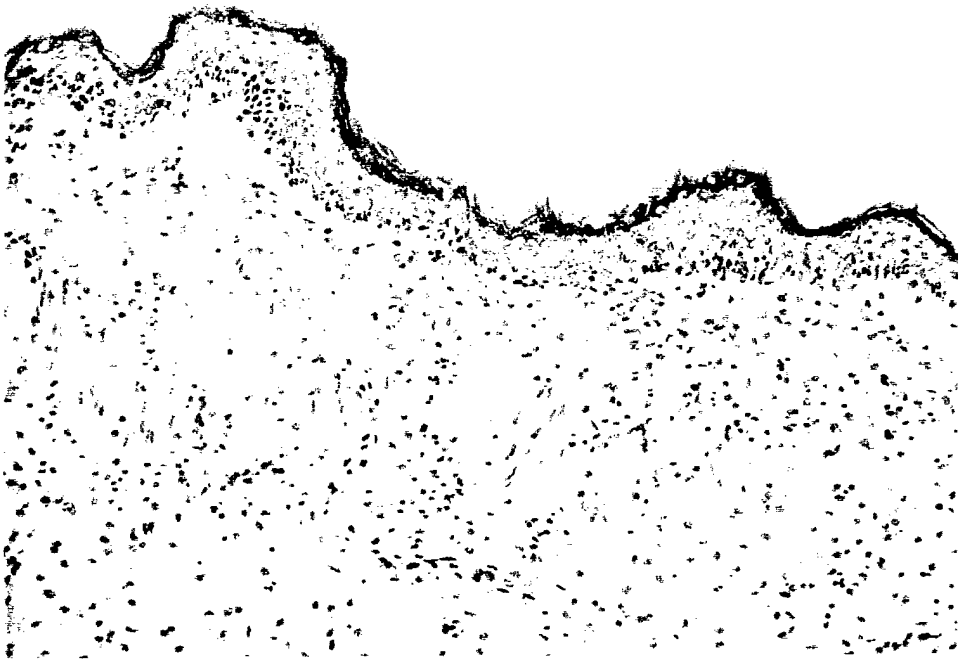


Animal #1 Dose Site D HD Dose - 0 μL

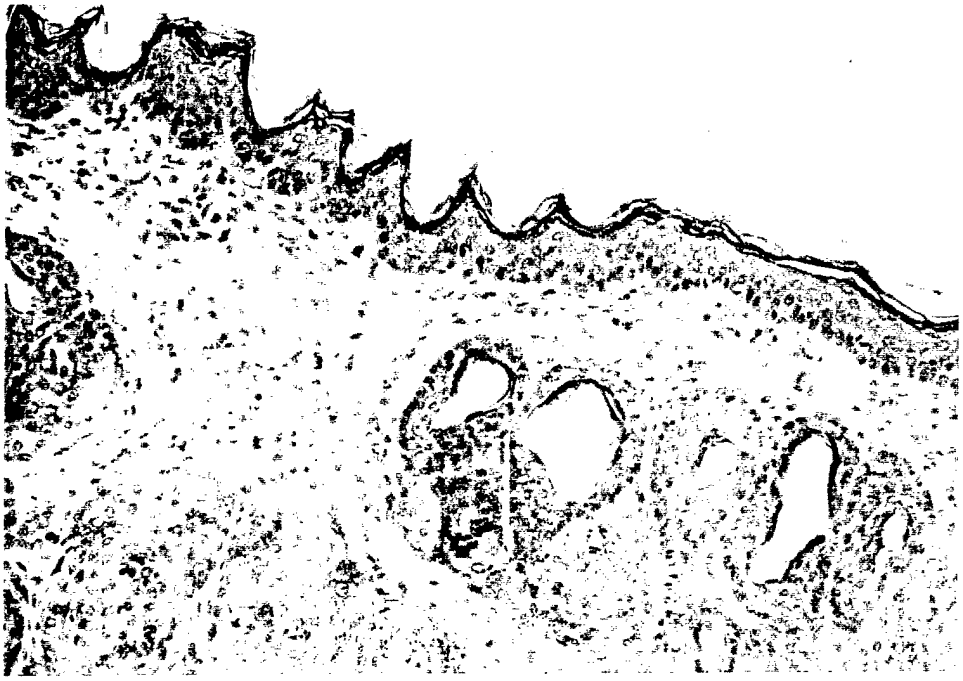
Animal #1 Dose Site C HD Dose - 0.25 μL



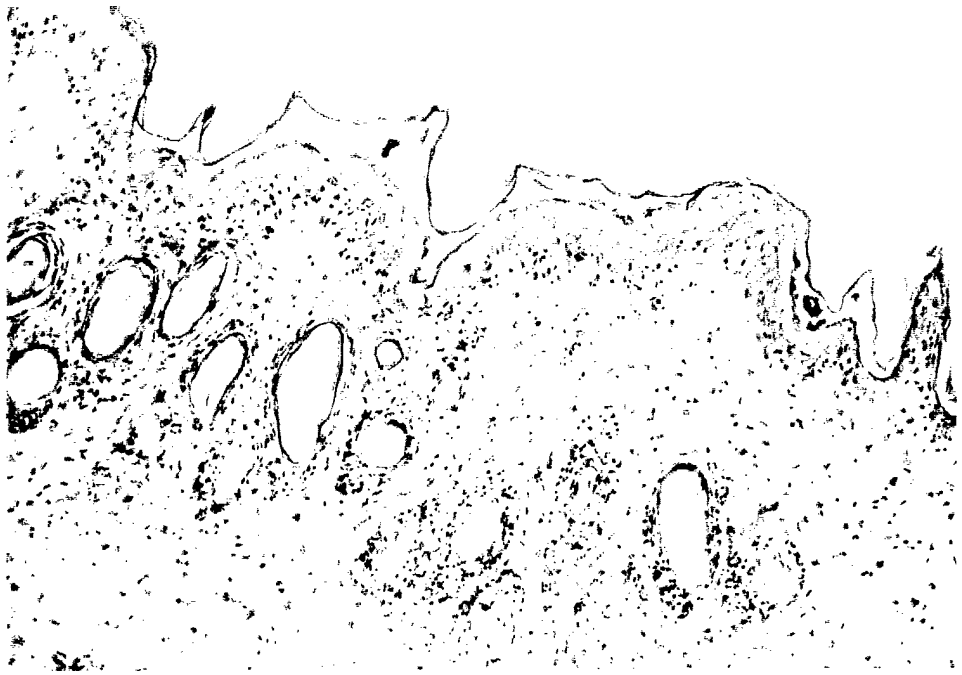
Animal #1 Dose Site B HD Dose - 0.5 μL



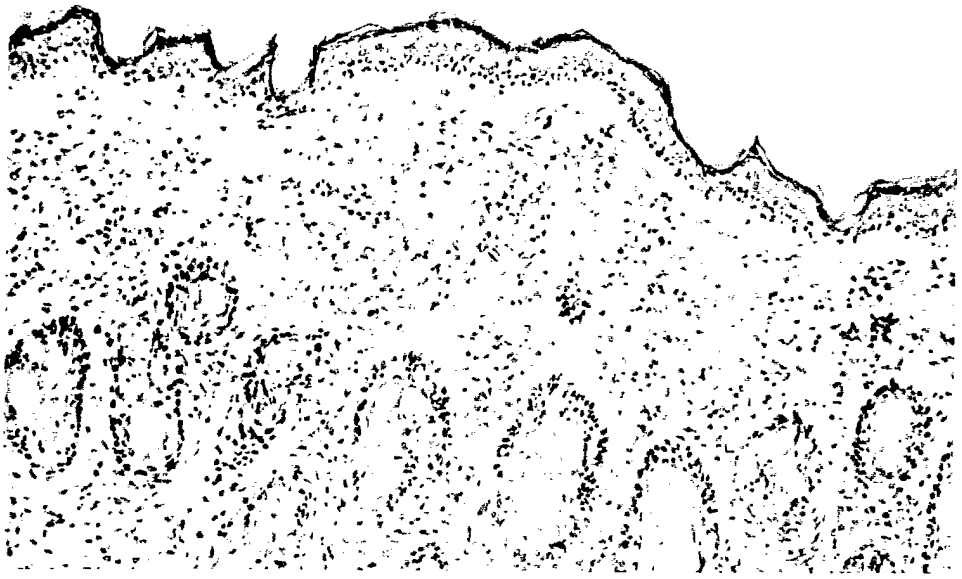
Animal #1 Dose Site E HD Dose - 1 μL



Animal #2 Dose Site D HD Dose - 0 μL

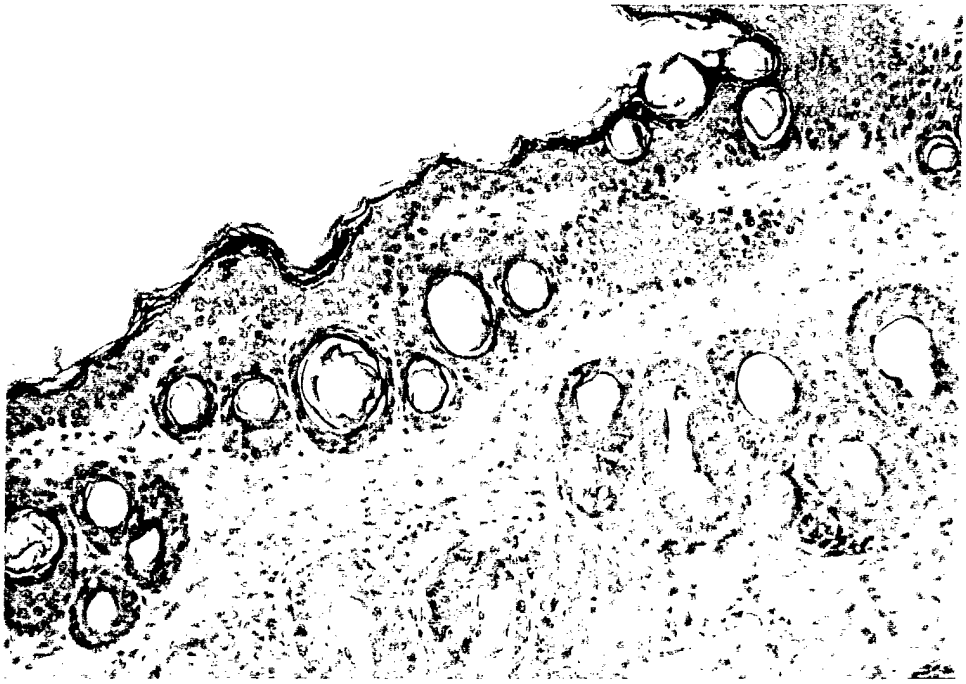


Animal #2 Dose Site F HD Dose - 0.25 μL



Animal #2 Dose Site A HD Dose - 0.5 μL

Animal #2 Dose Site E HD Dose - 1 μL

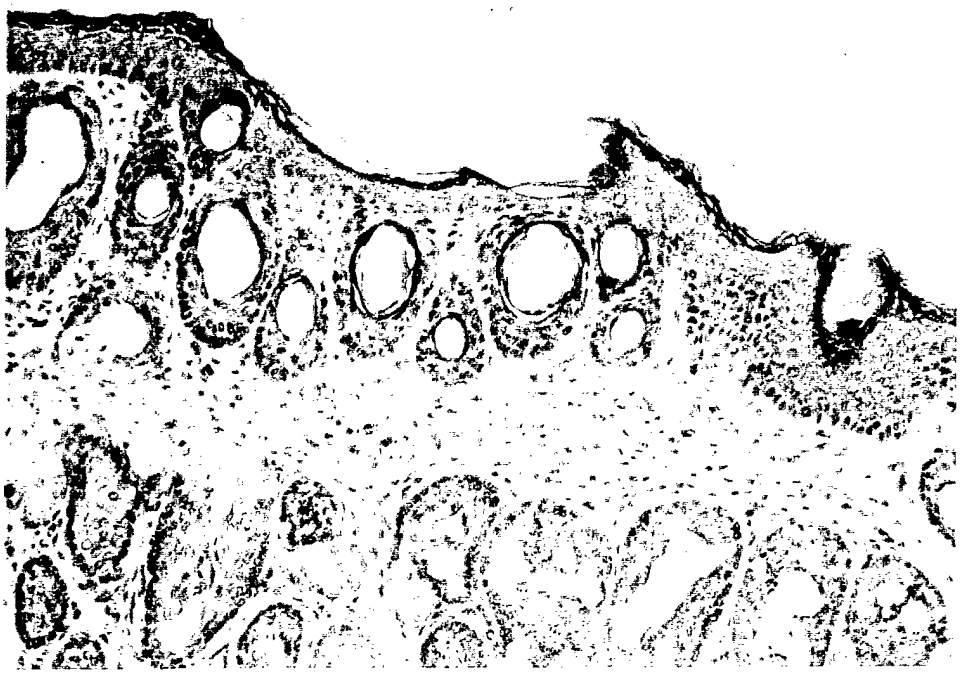


Animal #3 Dose Site A HD Dose - 0 μL

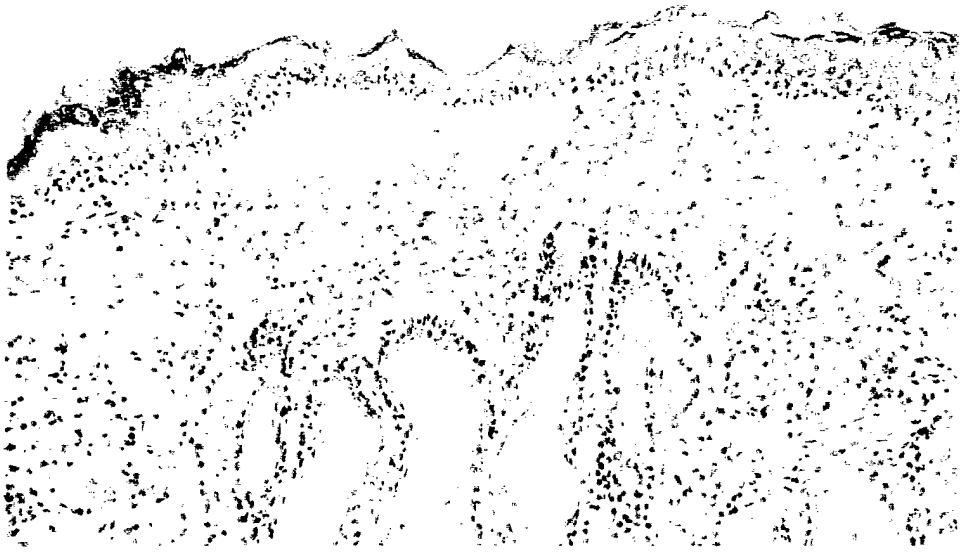
Animal #3 Dose Site D HD Dose - 0.25 μL

Animal #3 Dose Site E HD Dose - 0.5 μL

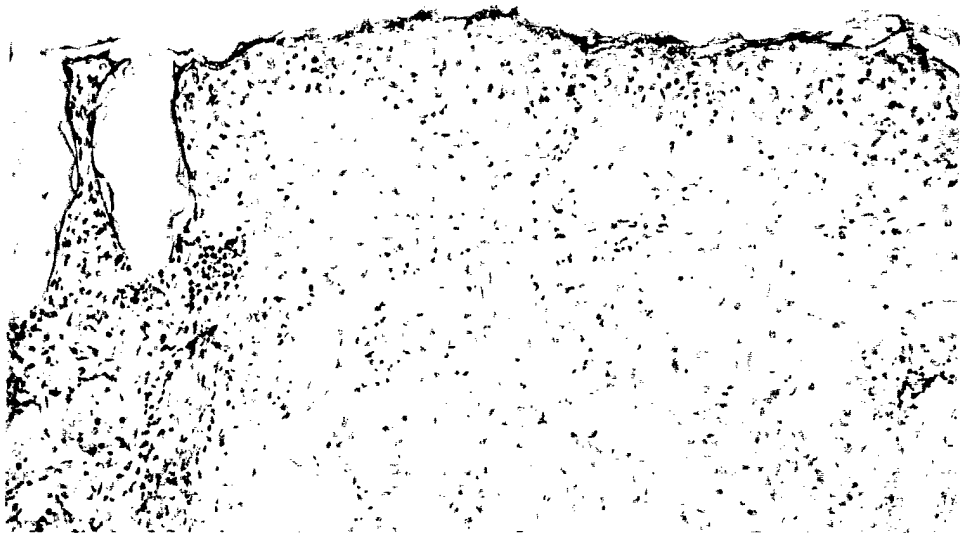
Animal #3 Dose Site F HD Dose - 1 μL



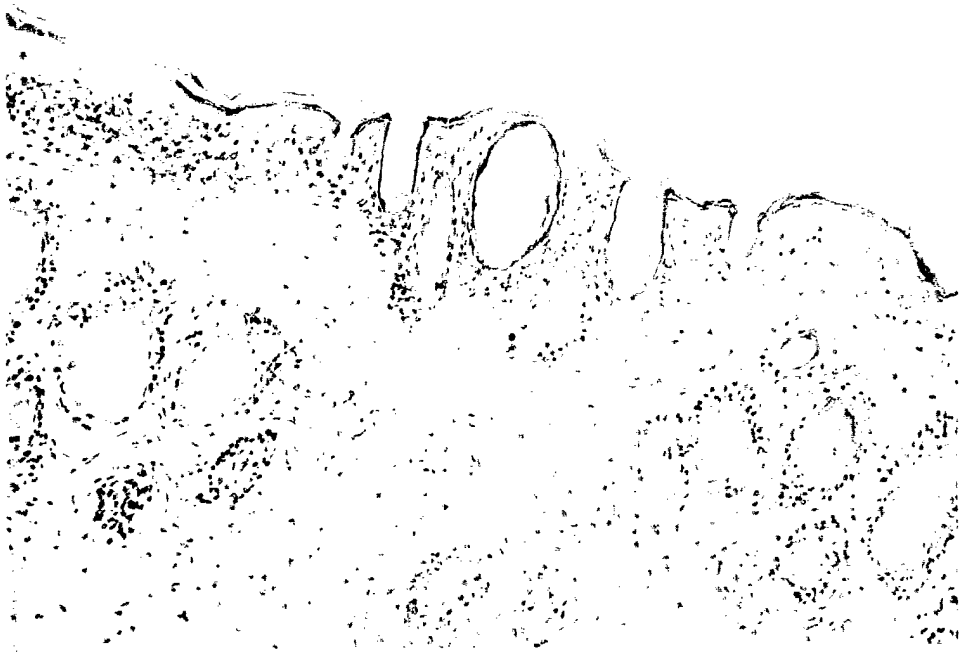
Animal #4 Dose Site A HD Dose - 0 μL



Animal #4 Dose Site B HD Dose - 0.25 μL

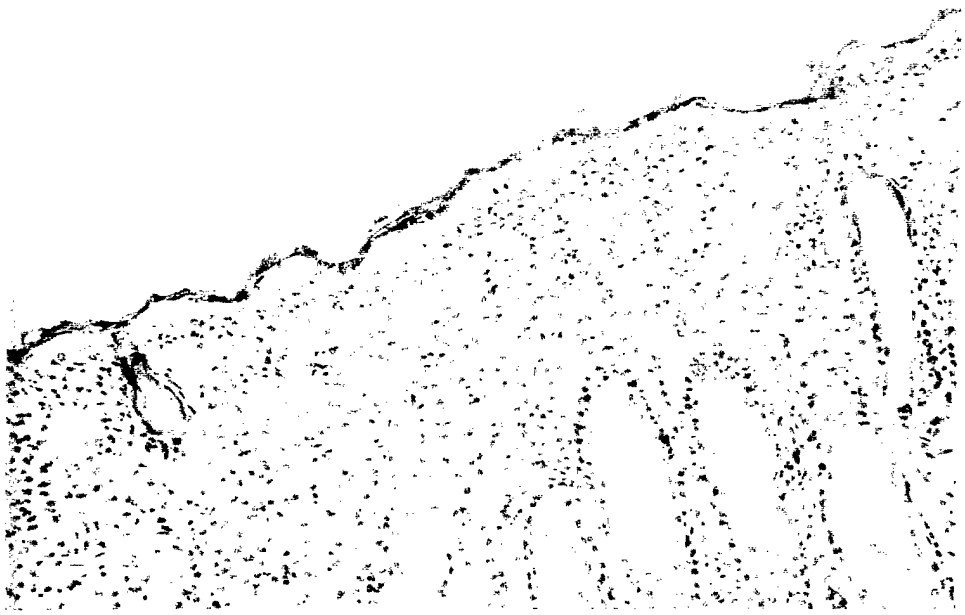


Animal #4 Dose Site C HD Dose - 0.5 μL

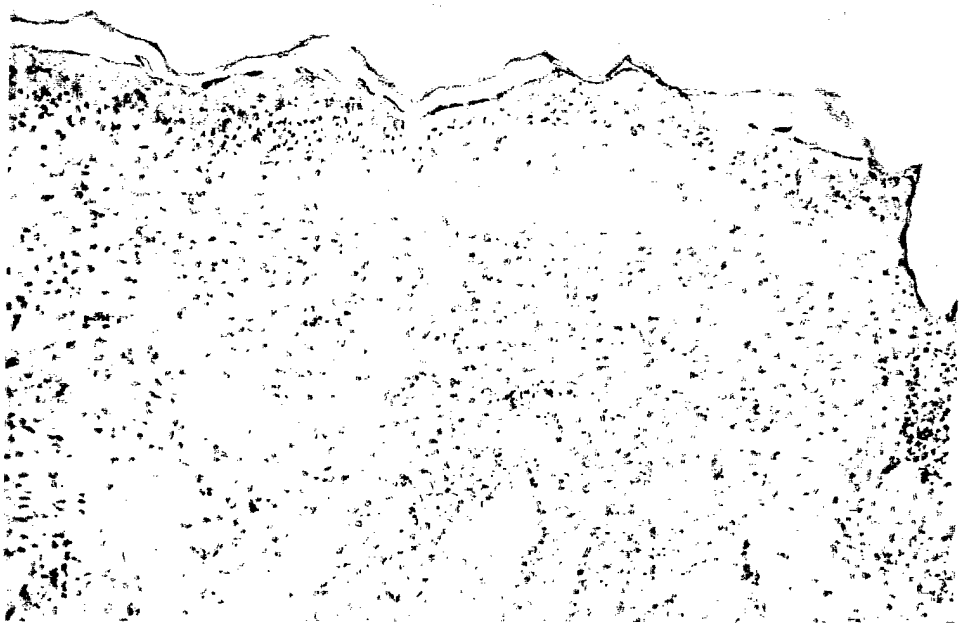


Animal #4 Dose Site F HD Dose - 1 μL

Animal #5 Dose Site E HD Dose - 0 μL

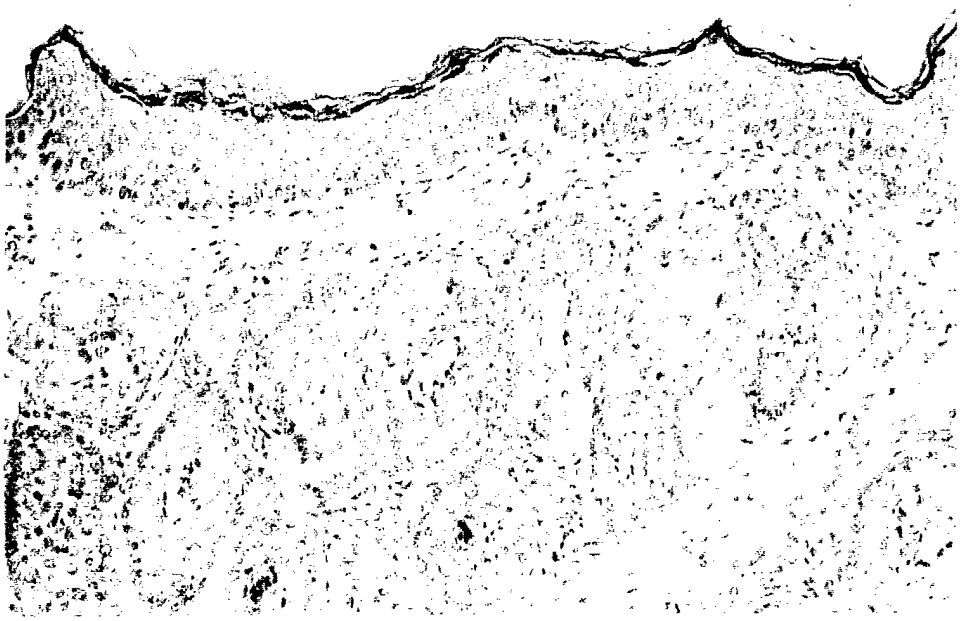


Animal #5 Dose Site A HD Dose - 0.25 μL



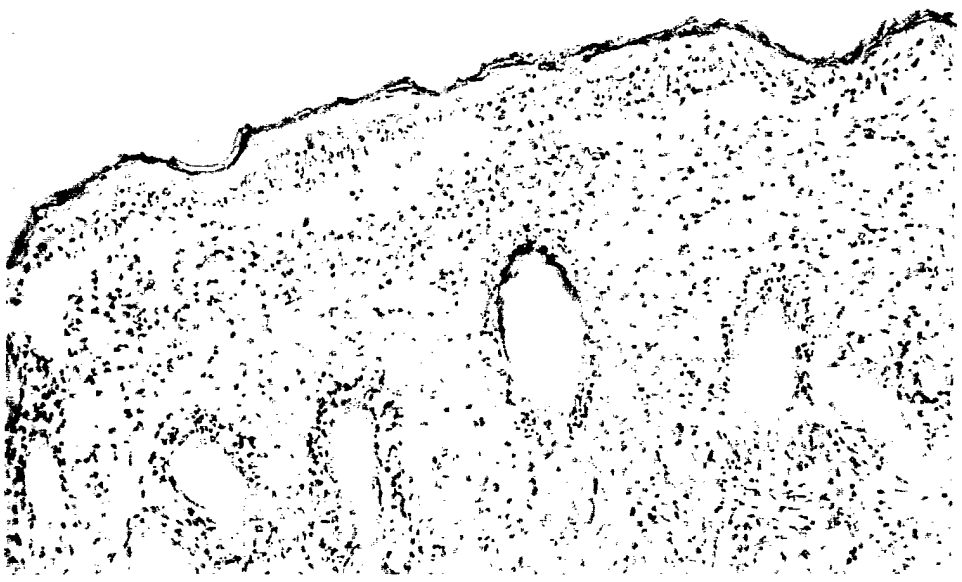
Animal #5 Dose Site F HD Dose - 0.5 μL

Animal #5 Dose Site D HD Dose - 1 μL

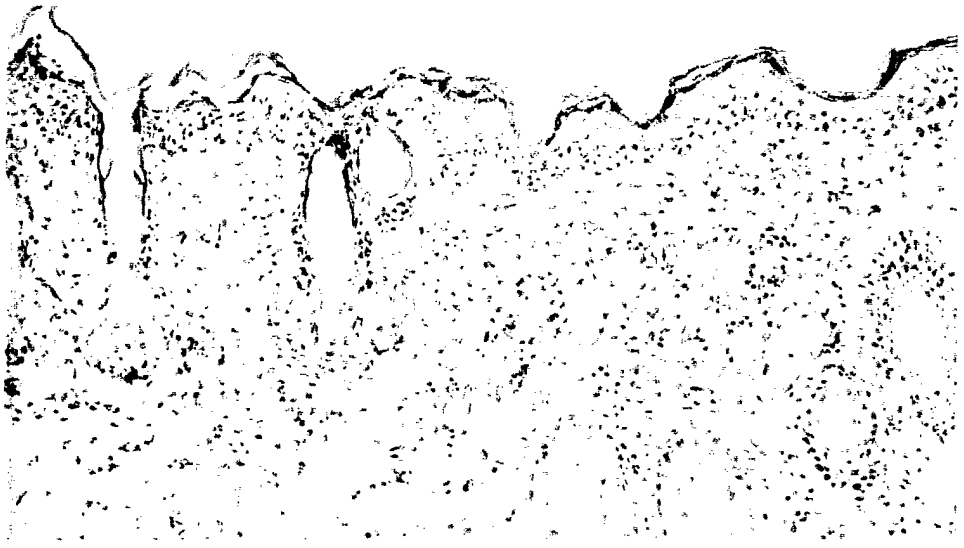


Animal #6 Dose Site F HD Dose - 0 μL

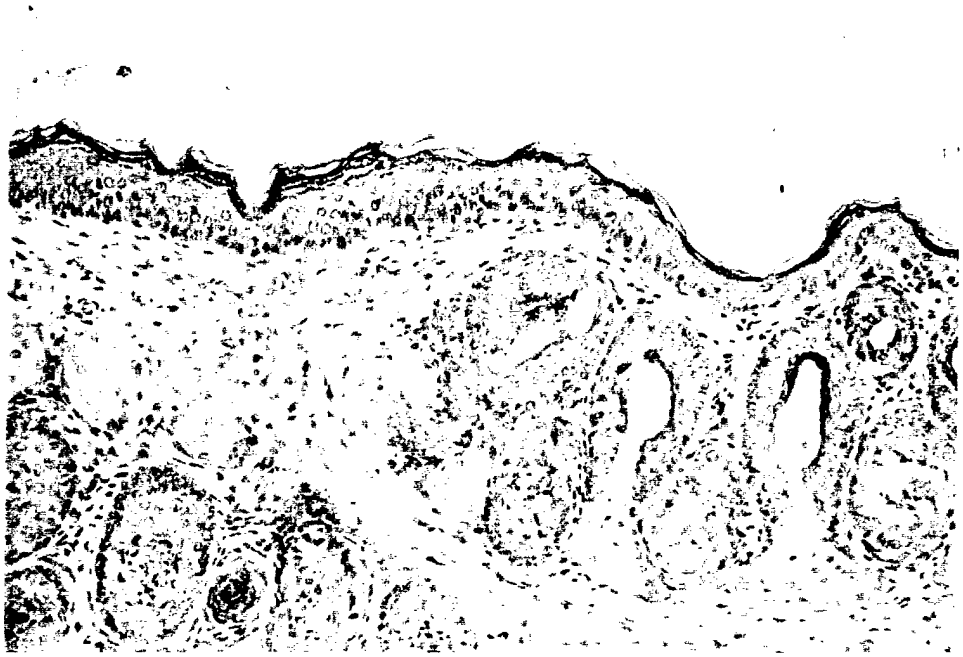
Animal #6 Dose Site E HD Dose - 0.25 μL



Animal #6 Dose Site D HD Dose - 0.5 μL

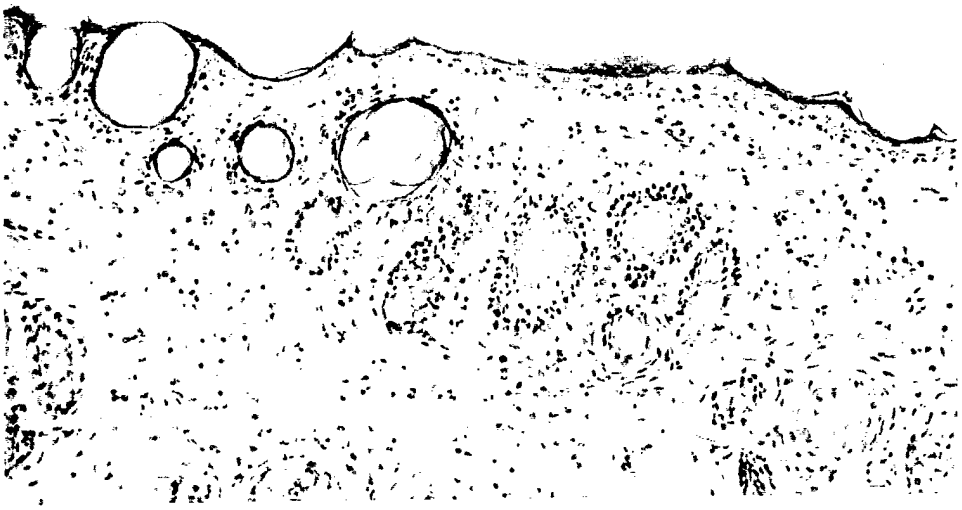


Animal #6 Dose Site B HD Dose - 1 μL

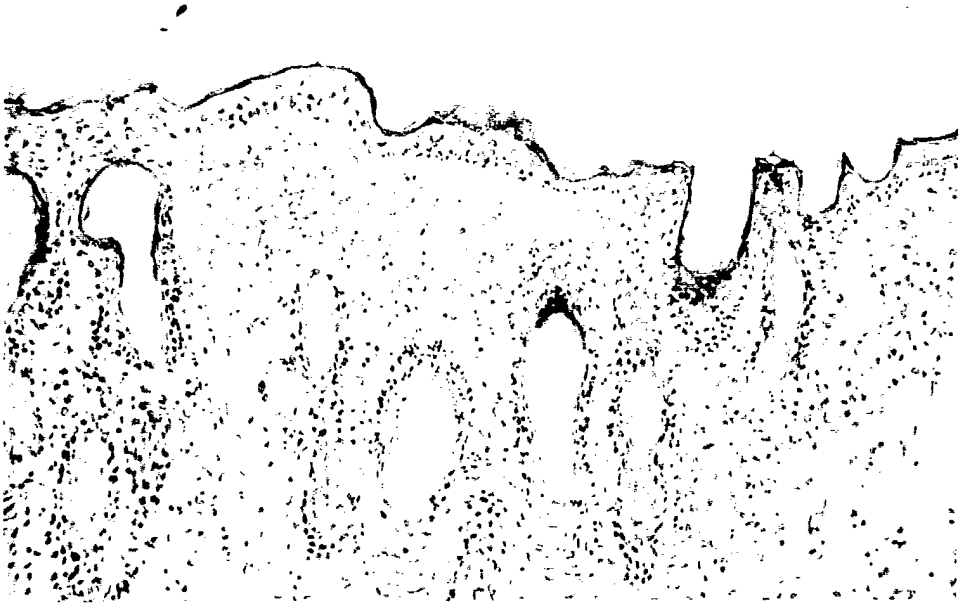


Animal #7 Dose Site F HD Dose - 0 μL

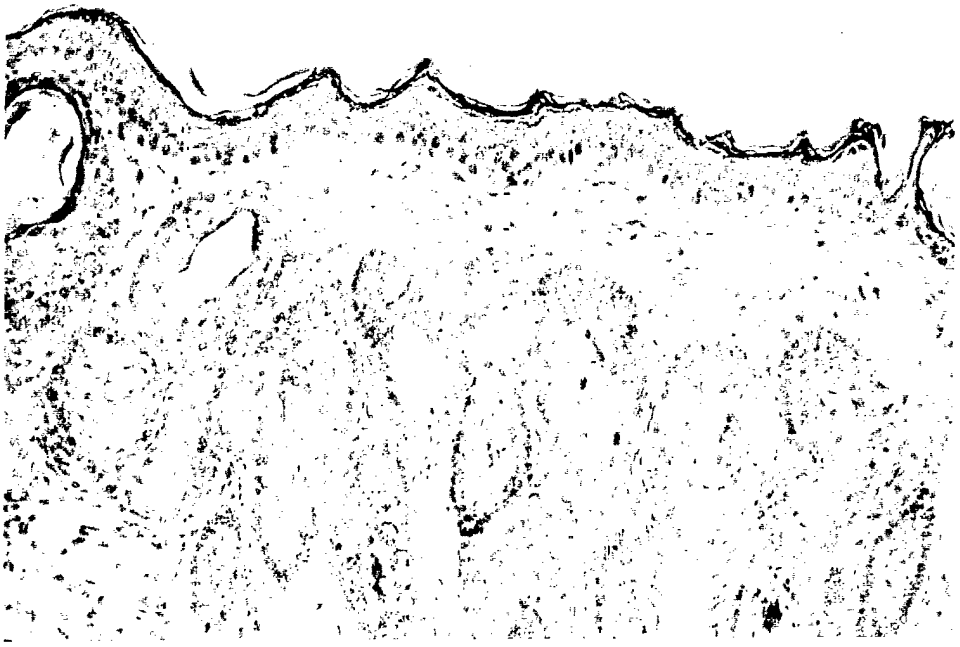
Animal #7 Dose Site C HD Dose - 0.25 μL



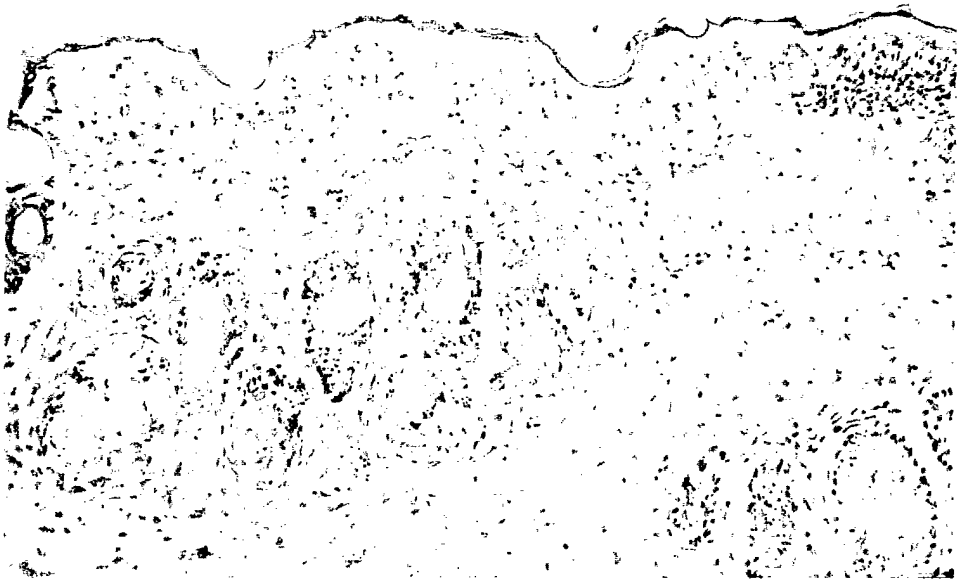
Animal #7 Dose Site B HD Dose - 0.5 μL



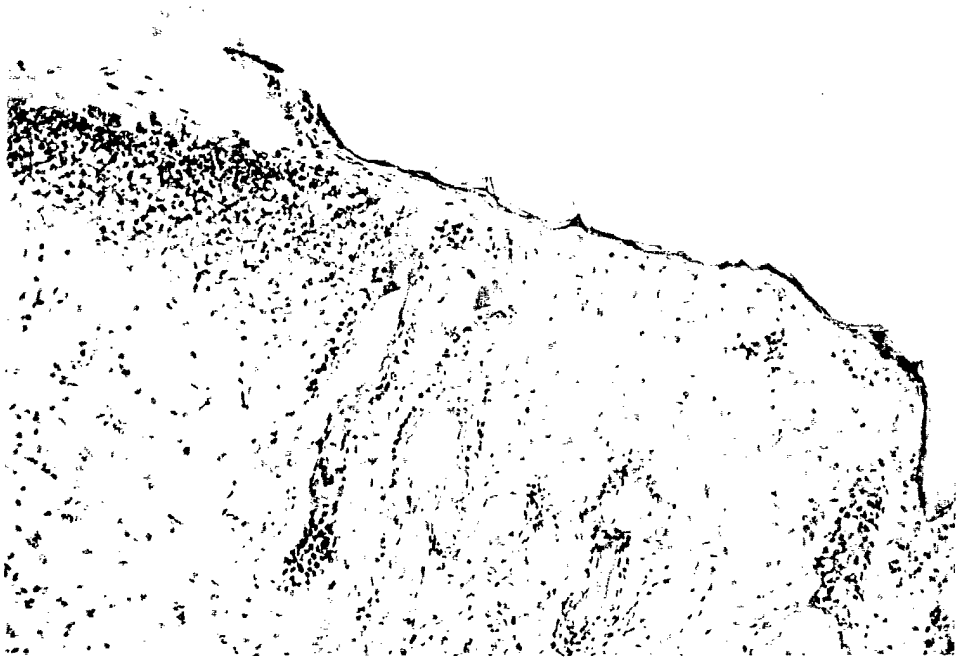
Animal #7 Dose Site A HD Dose - 1 μL



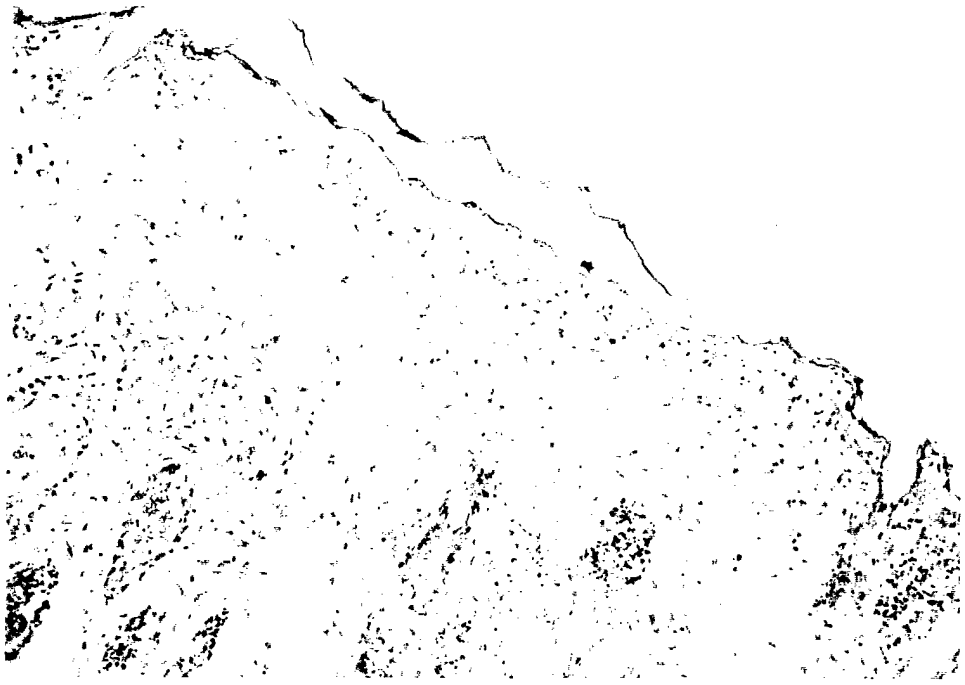
Animal #8 Dose Site D HD Dose - 0 μL



Animal #8 Dose Site F HD Dose - 0.25 μL



Animal #8 Dose Site A HD Dose - 0.5 μL



Animal #8 Dose Site E HD Dose - 1 μL

APPENDIX E

DATA FOR ANIMALS DOSED WITH VESICATING AND NON-VESICATING ALKYLATING AGENTS

HISTOPATHOLOGY KEY

EN - Epidermal Necrosis
FN - Follicular Necrosis
MB - Microblister
IE - Intracellular Edema
PE - Pustular Epidermitis

TABLE E-1. DATA OF ANIMAL DOSED WITH VESICATING AND NON-VESIATING AGENTS

		DOSE		ANIMAL			HISTOPATHOLOGY				
DATE	AGENT	(uL)	ABRAID	N	ID	SITE	EN	FN	MB	IE	PE
12/12/95	Mechloreth	10	No	1	**3187**	**A**	2	2	0	0	0
12/12/95	Mechloreth	10	No	2	**3188**	**C**	1	1	0	0	0
12/12/95	Mechloreth	10	No	3	**3182**	**D**	4	4	3	1	1
12/12/95	Mechloreth	10	No	4	**3185**	**B**	2	2	0	0	0
12/12/95	Mechloreth	10	No	5	**3190**	**A**	2	2	0	0	0
12/12/95	Mechloreth	10	No	6	**3184**	**C**	3	2	1	1	1
12/12/95	Mechloreth	10	No	7	**3189**	**D**	4	4	4	2	2
12/12/95	Mechloreth	10	No	8	**3183**	**B**	3	2	0	1	1
12/19/95	Mechloreth	10	No	9	**485**	**A**	1	1	0	0	0
12/19/95	Mechloreth	10	No	10	**484**	**C**	0	1	0	0	0
12/19/95	Mechloreth	10	No	11	**487**	**B**	4	4	3	1	0
12/19/95	Mechloreth	10	No	12	**482**	**A**	4	4	3	1	0
12/19/95	Mechloreth	10	No	13	**486**	**C**	0	1	0	1	0
12/19/95	Mechloreth	10	No	14	**481**	**D**	3	3	2	1	0
12/19/95	Mechloreth	10	No	15	**488**	**B**	2	3	1	1	1
3/27/96	Mechloreth	10	Yes	16	**326**	**A**	3	4	2	1	0
3/27/96	Mechloreth	10	Yes	17	**336**	**C**	4	4	2	1	0
3/27/96	Mechloreth	10	Yes	18	**334**	**B**	4	4	1	1	0
3/27/96	Mechloreth	10	Yes	19	**335**	**A**	4	4	2	1	1
3/27/96	Mechloreth	10	Yes	20	**333**	**C**	4	4	2	1	1
3/27/96	Mechloreth	10	Yes	21	**329**	**D**	4	4	2	2	1
3/27/96	Mechloreth	10	Yes	22	**331**	**B**	4	4	1	1	1
12/12/95	Thiotepa	5	Yes	1	**3187**	**B**	2	1	1	0	1
12/12/95	Thiotepa	5	Yes	2	**3188**	**A**	0	0	0	0	0
12/12/95	Thiotepa	5	Yes	3	**3182**	**C**	1	1	0	0	0
12/12/95	Thiotepa	5	Yes	4	**3185**	**D**	2	1	0	0	1
12/12/95	Thiotepa	5	Yes	5	**3190**	**B**	1	1	0	0	1
12/12/95	Thiotepa	5	Yes	6	**3184**	**A**	1	1	0	0	1
12/12/95	Thiotepa	5	Yes	7	**3189**	**C**	1	1	0	0	1
12/12/95	Thiotepa	5	Yes	8	**3183**	**D**	1	1	0	0	1
12/19/95	Thiotepa	5	Yes	9	**485**	**B**	2	1	0	0	0
12/19/95	Thiotepa	5	Yes	10	**484**	**A**	0	0	0	0	0
12/19/95	Thiotepa	5	Yes	11	**487**	**D**	1	1	0	0	0
12/19/95	Thiotepa	5	Yes	12	**482**	**B**	0	1	0	0	1
12/19/95	Thiotepa	5	Yes	13	**486**	**A**	0	1	0	0	0
12/19/95	Thiotepa	5	Yes	14	**481**	**C**	3	3	1	1	3
12/19/95	Thiotepa	5	Yes	15	**488**	**D**	1	1	0	0	0
4/3/95	Cis-plat.	5	No	1	**476**	**D**	0	0	0	0	0
4/3/95	Cis-plat.	5	No	2	**477**	**B**	0	0	0	0	0
4/3/95	Cis-plat.	5	No	3	**479**	**D**	0	0	0	0	0
4/3/95	Cis-plat.	5	No	4	**480**	**A**	0	0	0	0	0
12/12/95	Cis-plat.	5	Yes	5	**3187**	**C**	1	1	0	0	1
12/12/95	Cis-plat.	5	Yes	6	**3188**	**D**	1	1	0	0	1
12/12/95	Cis-plat.	5	Yes	7	**3182**	**B**	0	1	0	0	1
12/12/95	Cis-plat.	5	Yes	8	**3185**	**A**	1	1	1	1	1

TABLE E-1.
(Continued)

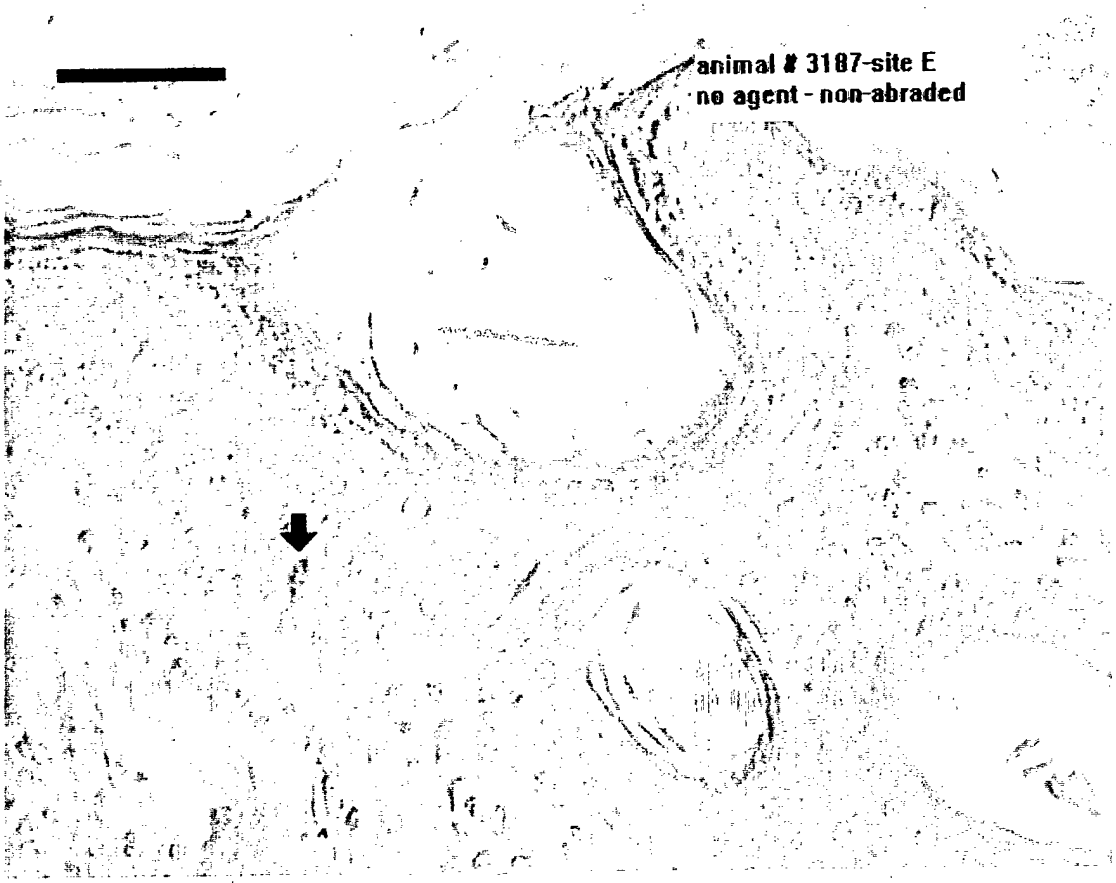
DATE	AGENT	DOSE (uL)	ABRAID	ANIMAL N	ID	SITE	HISTOPATHOLOGY EN	FN	MB	IE	PE
12/12/95	Cis-plat.	5	Yes	9	**3190**	**C**	1	1	0	0	1
12/12/95	Cis-plat.	5	Yes	10	**3184**	**D**	0	1	0	0	0
12/12/95	Cis-plat.	5	Yes	11	**3189**	**B**	2	2	0	1	1
12/12/95	Cis-plat.	5	Yes	12	**3183**	**A**	0	1	0	0	1
12/19/95	Cis-plat.	5	Yes	13	**485**	**C**	1	1	0	0	1
12/19/95	Cis-plat.	5	Yes	14	**484**	**D**	2	1	0	0	0
12/19/95	Cis-plat.	5	Yes	15	**487**	**A**	2	2	0	1	0
12/19/95	Cis-plat.	5	Yes	16	**482**	**C**	1	1	0	0	0
12/19/95	Cis-plat.	5	Yes	17	**486**	**D**	1	1	0	0	0
12/19/95	Cis-plat.	5	Yes	18	**481**	**B**	2	1	0	1	3
12/19/95	Cis-plat.	5	Yes	19	**488**	**A**	1	1	0	0	0
4/3/95	Melphalan	10	No	1	**476**	**B**	0	0	0	0	0
4/3/95	Melphalan	10	No	2	**477**	**A**	0	0	0	0	0
4/3/95	Melphalan	10	No	3	**479**	**C**	0	0	0	0	0
4/3/95	Melphalan	10	No	4	**480**	**B**	0	0	0	0	0
12/12/95	Melphalan	10	Yes	5	**3187**	**D**	1	1	0	0	1
12/12/95	Melphalan	10	Yes	6	**3188**	**B**	1	1	0	0	1
12/12/95	Melphalan	10	Yes	7	**3182**	**A**	1	1	0	0	0
12/12/95	Melphalan	10	Yes	8	**3185**	**C**	1	1	0	0	1
12/12/95	Melphalan	10	Yes	9	**3190**	**D**	1	1	0	0	1
12/12/95	Melphalan	10	Yes	10	**3184**	**B**	1	1	0	0	1
12/12/95	Melphalan	10	Yes	11	**3189**	**A**	1	1	0	0	1
12/12/95	Melphalan	10	Yes	12	**3183**	**C**	1	1	0	0	1
12/19/95	Melphalan	10	Yes	13	**485**	**D**	1	1	0	0	1
12/19/95	Melphalan	10	Yes	14	**484**	**B**	2	1	0	0	0
12/19/95	Melphalan	10	Yes	15	**487**	**C**	0	0	0	0	0
12/19/95	Melphalan	10	Yes	16	**482**	**D**	1	1	0	0	1
12/19/95	Melphalan	10	Yes	17	**486**	**B**	1	1	0	0	1
12/19/95	Melphalan	10	Yes	18	**481**	**A**	0	1	0	0	0
12/19/95	Melphalan	10	Yes	19	**488**	**C**	1	1	0	0	0
4/3/95	CEES	5	No	1	**476**	**A**	4	3	3	2	0
4/3/95	CEES	5	No	2	**477**	**C**	4	3	2	2	0
4/3/95	CEES	5	No	3	**479**	**A**	4	3	3	2	0
4/3/95	CEES	5	No	4	**480**	**D**	4	3	3	2	0
3/27/96	CEES	5	No	5	**326**	**B**	4	3	3	1	0
3/27/96	CEES	5	No	6	**336**	**A**	3	3	3	2	0
3/27/96	CEES	5	No	7	**334**	**D**	4	4	2	2	0
3/27/96	CEES	5	No	8	**335**	**B**	4	3	4	1	0
3/27/96	CEES	5	No	9	**333**	**A**	4	3	3	1	1
3/27/96	CEES	5	No	10	**329**	**C**	4	3	3	1	1
3/27/96	CEES	5	No	11	**331**	**D**	4	4	3	1	0
4/3/95	HD	0.5	No	1	**476**	**F**	4	4	3	2	0
4/3/95	HD	0.5	No	2	**477**	**F**	4	4	3	1	1
4/3/95	HD	0.5	No	3	**479**	**F**	4	4	2	2	0
4/3/95	HD	0.5	No	4	**480**	**F**	4	4	3	2	0
12/12/95	HD	0.5	No	5	**3187**	**F**	4	4	3	1	2

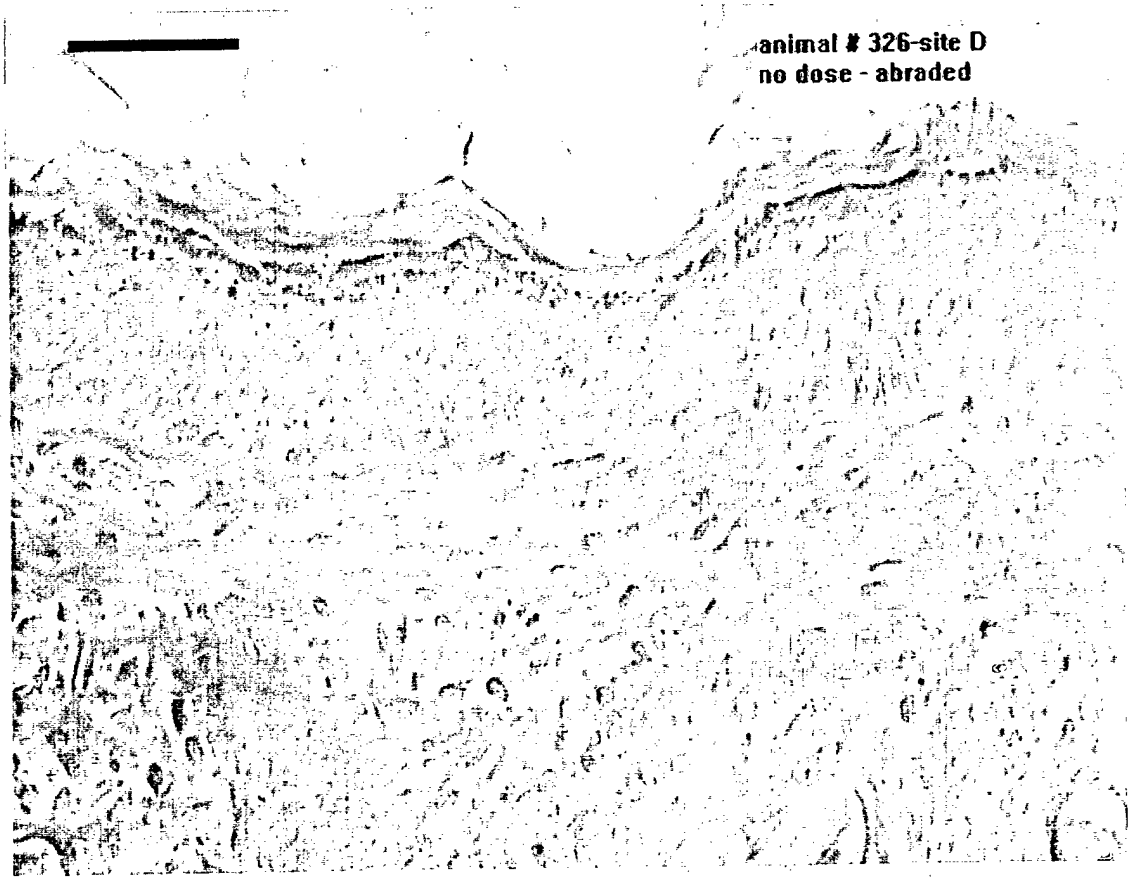
TABLE E-1.
(Continued)

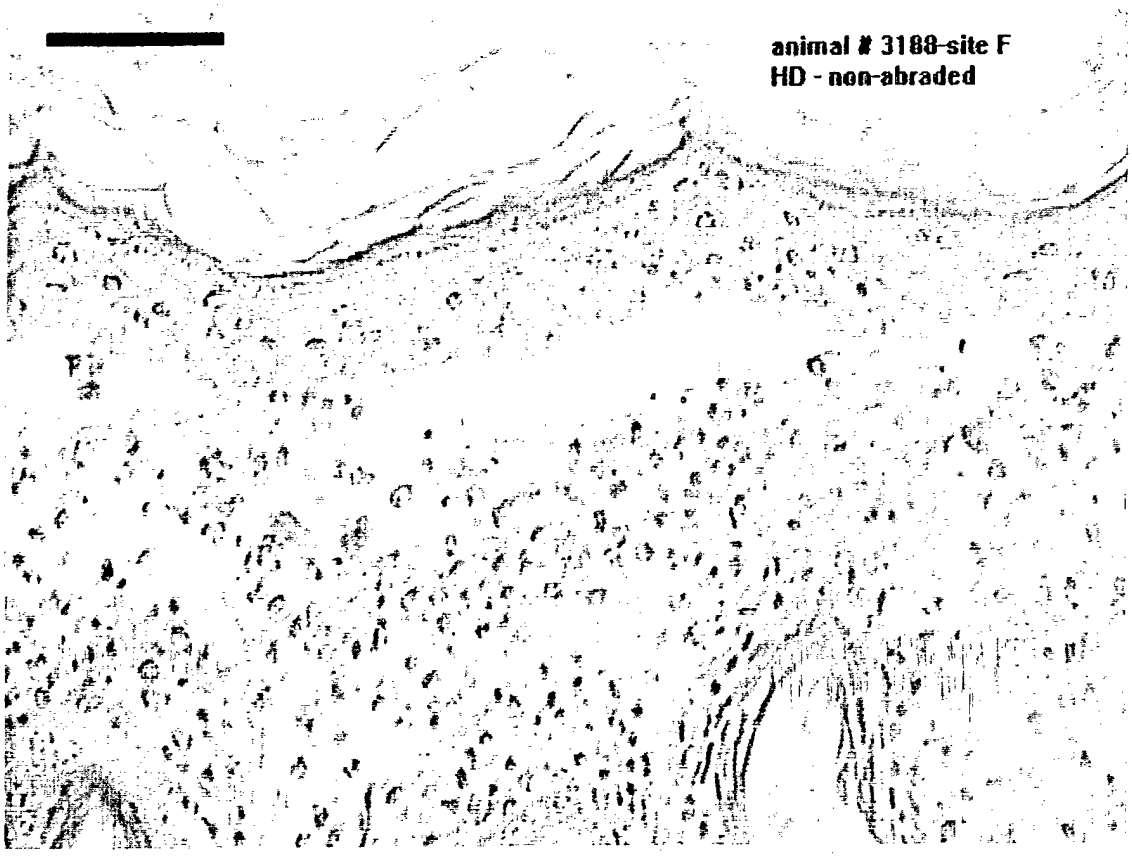
DATE	AGENT	DOSE (uL)	ABRAID	ANIMAL N	ID	SITE	HISTOPATHOLOGY EN	FN	MB	IE	PE
12/12/95	HD	0.5	No	6	**3188**	**F**	4	4	3	2	1
12/12/95	HD	0.5	No	7	**3182**	**F**	4	4	3	1	1
12/12/95	HD	0.5	No	8	**3185**	**F**	4	4	4	2	1
12/12/95	HD	0.5	No	9	**3190**	**F**	4	4	3	2	0
12/12/95	HD	0.5	No	10	**3184**	**F**	4	4	2	1	1
12/12/95	HD	0.5	No	11	**3189**	**G**	4	4	3	2	1
12/12/95	HD	0.5	No	12	**3183**	**F**	4	4	2	2	0
12/19/95	HD	0.5	No	13	**485**	**F**	4	4	3	2	0
12/19/95	HD	0.5	No	14	**484**	**F**	4	3	3	1	0
12/19/95	HD	0.5	No	15	**487**	**F**	4	4	3	1	1
12/19/95	HD	0.5	No	16	**482**	**F**	4	4	3	1	1
12/19/95	HD	0.5	No	17	**486**	**F**	4	4	2	1	0
12/19/95	HD	0.5	No	18	**481**	**F**	4	4	3	1	1
12/19/95	HD	0.5	No	19	**488**	**F**	4	4	2	1	1
3/27/96	HD	0.5	No	20	**326**	**H**	3	4	3	2	0
3/27/96	HD	0.5	No	21	**336**	**H**	3	4	2	1	0
3/27/96	HD	0.5	No	22	**334**	**H**	3	4	1	2	0
3/27/96	HD	0.5	No	23	**335**	**H**	3	4	2	2	1
3/27/96	HD	0.5	No	24	**333**	**H**	3	4	2	1	0
3/27/96	HD	0.5	No	25	**329**	**H**	3	4	2	2	1
3/27/96	HD	0.5	No	26	**331**	**H**	4	4	3	1	0
4/3/95	Ethanol	5	No	1	**476**	**C**	0	0	0	0	0
4/3/95	Ethanol	5	No	2	**477**	**D**	0	0	0	0	0
4/3/95	Ethanol	5	No	3	**479**	**B**	0	0	0	0	0
4/3/95	Ethanol	5	No	4	**480**	**C**	0	0	0	0	0
12/12/95	Ethanol	5	Yes	5	**3187**	**G**	0	0	0	0	0
12/12/95	Ethanol	5	Yes	6	**3188**	**G**	0	1	0	0	1
12/12/95	Ethanol	5	Yes	7	**3182**	**G**	0	1	0	0	1
12/12/95	Ethanol	5	Yes	8	**3185**	**G**	1	1	0	0	1
12/12/95	Ethanol	5	Yes	9	**3190**	**G**	1	1	0	0	0
12/12/95	Ethanol	5	Yes	10	**3184**	**G**	1	1	0	0	0
12/12/95	Ethanol	5	Yes	11	**3189**	**F**	1	1	0	0	0
12/12/95	Ethanol	5	Yes	12	**3183**	**G**	1	1	0	0	0
12/19/95	Ethanol	5	Yes	13	**485**	**G**	0	1	0	0	1
12/19/95	Ethanol	5	Yes	14	**484**	**G**	0	0	0	0	0
12/19/95	Ethanol	5	Yes	15	**487**	**G**	0	0	0	0	0
12/19/95	Ethanol	5	Yes	16	**482**	**G**	0	0	0	0	0
12/19/95	Ethanol	5	Yes	17	**486**	**G**	1	1	0	1	0
12/19/95	Ethanol	5	Yes	18	**481**	**G**	1	1	0	0	0
12/19/95	Ethanol	5	Yes	19	**488**	**G**	0	0	0	0	0
3/27/96	Ethanol	5	Yes	20	**326**	**G**	1	1	0	0	1
3/27/96	Ethanol	5	Yes	21	**336**	**G**	1	1	0	0	0
3/27/96	Ethanol	5	Yes	22	**334**	**G**	1	1	0	0	1
3/27/96	Ethanol	5	Yes	23	**335**	**G**	1	1	0	0	1

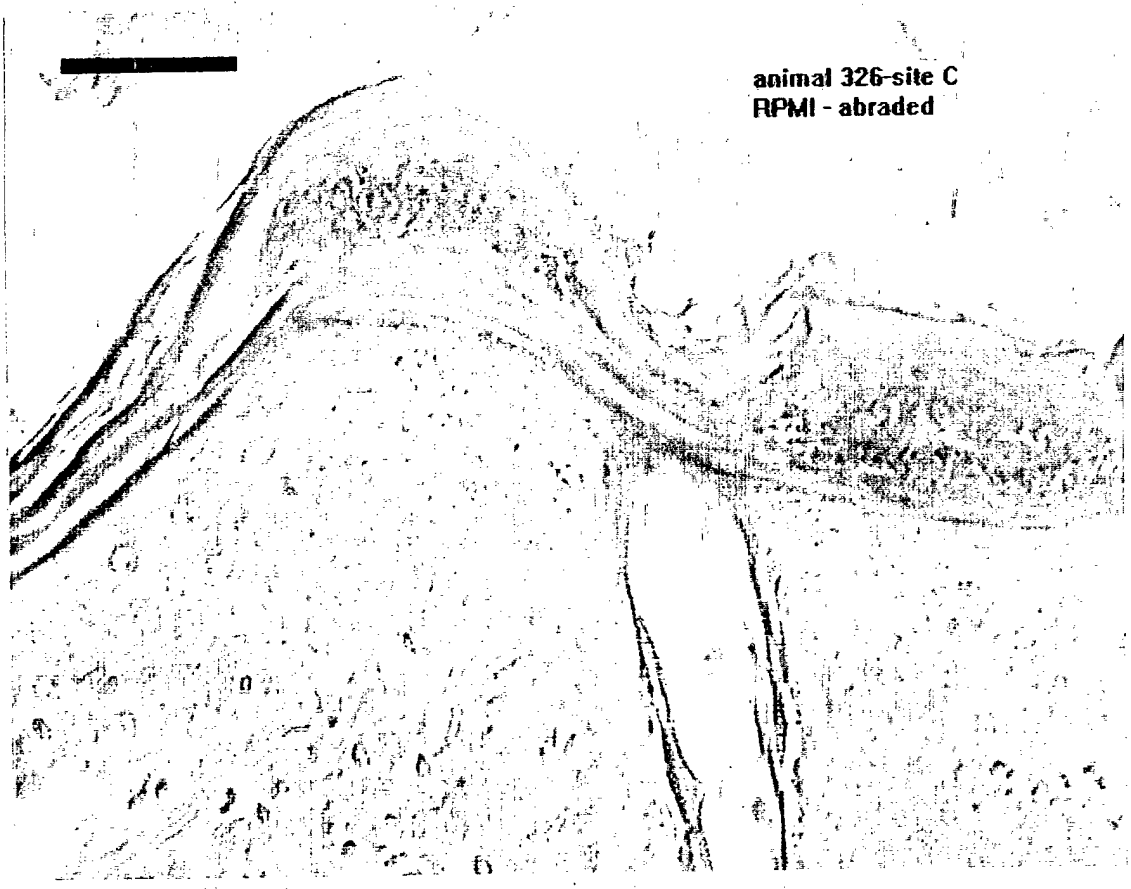
TABLE E-1.
(Continued)

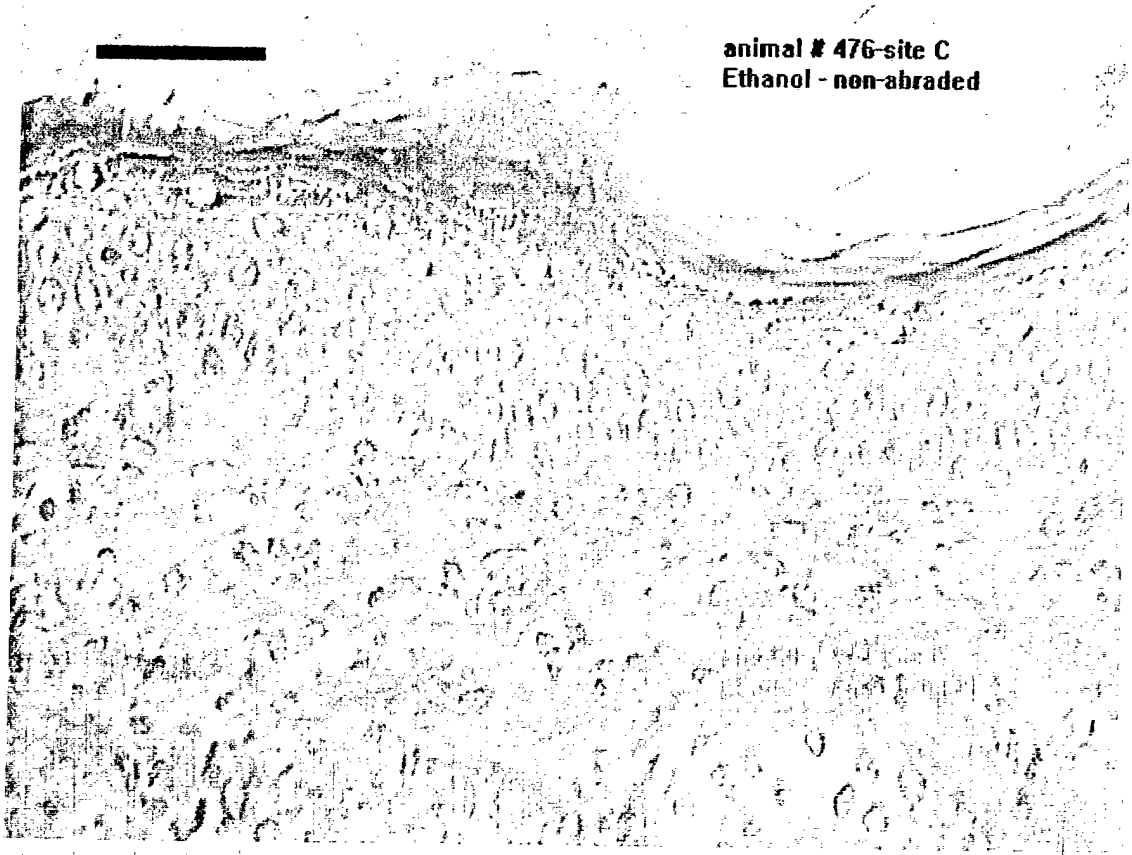
		DOSE		ANIMAL			HISTOPATHOLOGY				
DATE	AGENT	(uL)	ABRAID	N	ID	SITE	EN	FN	MB	IE	PE
3/27/96	Ethanol	5	Yes	24	**333**	**G**	2	1	0	0	1
3/27/96	Ethanol	5	Yes	25	**329**	**G**	0	1	0	0	0
3/27/96	Ethanol	5	Yes	26	**331**	**G**	1	1	0	0	0
4/3/95	None	0	No	1	**476**	**E**	0	0	0	0	0
4/3/95	None	0	No	2	**477**	**F**	0	0	0	0	0
4/3/95	None	0	No	3	**479**	**E**	0	0	0	0	0
4/3/95	None	0	No	4	**480**	**E**	0	0	0	0	0
12/12/95	None	0	No	5	**3187**	**E**	1	1	0	0	0
12/12/95	None	0	No	6	**3188**	**E**	1	1	0	0	0
12/12/95	None	0	No	7	**3182**	**E**	0	0	0	0	0
12/12/95	None	0	No	8	**3185**	**E**	1	1	0	0	0
12/12/95	None	0	No	9	**3190**	**E**	1	1	0	1	0
12/12/95	None	0	No	10	**3184**	**E**	1	1	0	0	0
12/12/95	None	0	No	11	**3189**	**E**	0	0	0	0	1
12/12/95	None	0	No	12	**3183**	**E**	0	1	0	0	0
12/19/95	None	0	No	13	**485**	**E**	2	3	2	0	0
12/19/95	None	0	No	14	**484**	**E**	0	1	0	0	0
12/19/95	None	0	No	15	**487**	**E**	0	0	0	0	0
12/19/95	None	0	No	16	**482**	**E**	0	0	0	0	0
12/19/95	None	0	No	17	**486**	**E**	0	0	0	0	0
12/19/95	None	0	No	18	**481**	**E**	0	1	0	0	0
12/19/95	None	0	No	19	**488**	**E**	1	1	0	0	0
3/27/96	None	0	No	20	**326**	**E**	1	0	0	0	0
3/27/96	None	0	No	21	**336**	**E**	1	1	0	0	0
3/27/96	None	0	No	22	**334**	**E**	0	0	0	0	0
3/27/96	None	0	No	23	**335**	**E**	1	0	0	0	0
3/27/96	None	0	No	24	**333**	**E**	1	1	0	0	0
3/27/96	None	0	No	25	**329**	**E**	1	1	0	0	0
3/27/96	None	0	No	26	**331**	**E**	1	1	0	0	0
3/27/96	None	0	Yes	27	**326**	**D**	1	0	0	0	1
3/27/96	None	0	Yes	28	**336**	**B**	0	0	0	0	1
3/27/96	None	0	Yes	29	**334**	**C**	1	1	0	0	1
3/27/96	None	0	Yes	30	**335**	**D**	1	1	0	0	0
3/27/96	None	0	Yes	31	**333**	**B**	1	1	0	0	0
3/27/96	None	0	Yes	32	**329**	**A**	0	0	0	0	1
3/27/96	None	0	Yes	33	**331**	**C**	1	1	0	0	1
3/27/96	RPMI	5	Yes	1	**326**	**C**	0	0	0	0	1
3/27/96	RPMI	5	Yes	2	**336**	**D**	0	0	0	0	0
3/27/96	RPMI	5	Yes	3	**334**	**A**	1	1	0	0	0
3/27/96	RPMI	5	Yes	4	**335**	**C**	1	1	0	0	1
3/27/96	RPMI	5	Yes	5	**333**	**D**	1	1	0	0	0
3/27/96	RPMI	5	Yes	6	**329**	**B**	1	1	0	0	1
3/27/96	RPMI	5	Yes	7	**331**	**A**	0	0	0	0	0

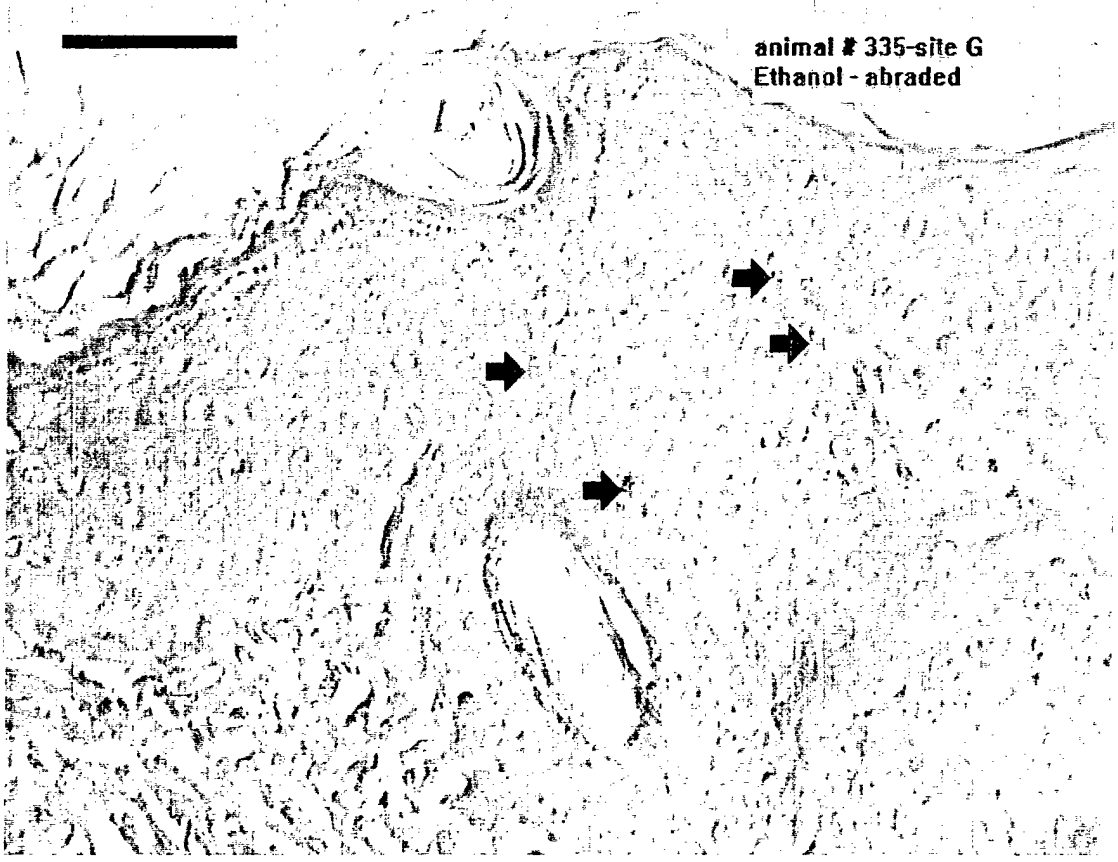

animal # 3187-site E
no agent - non-abraded

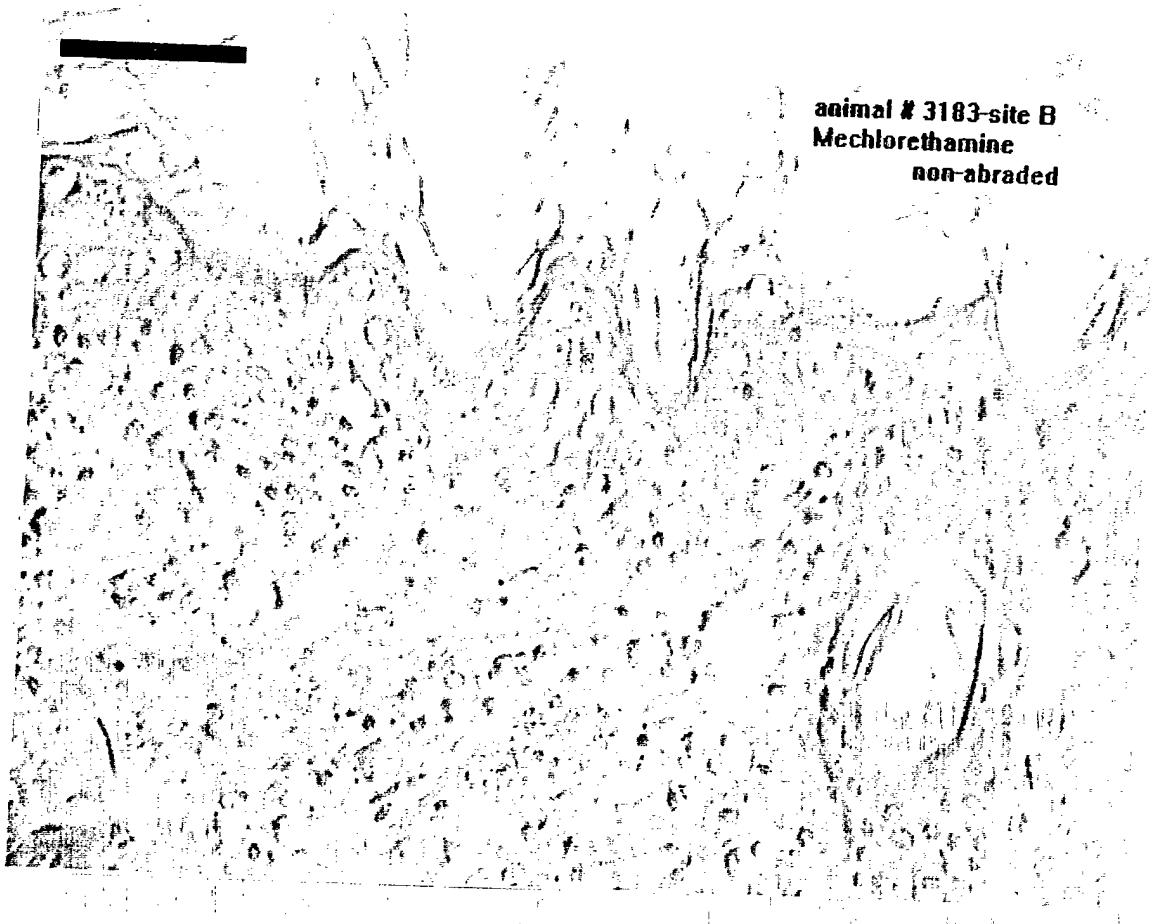

animal # 326-site D
no dose - abraded

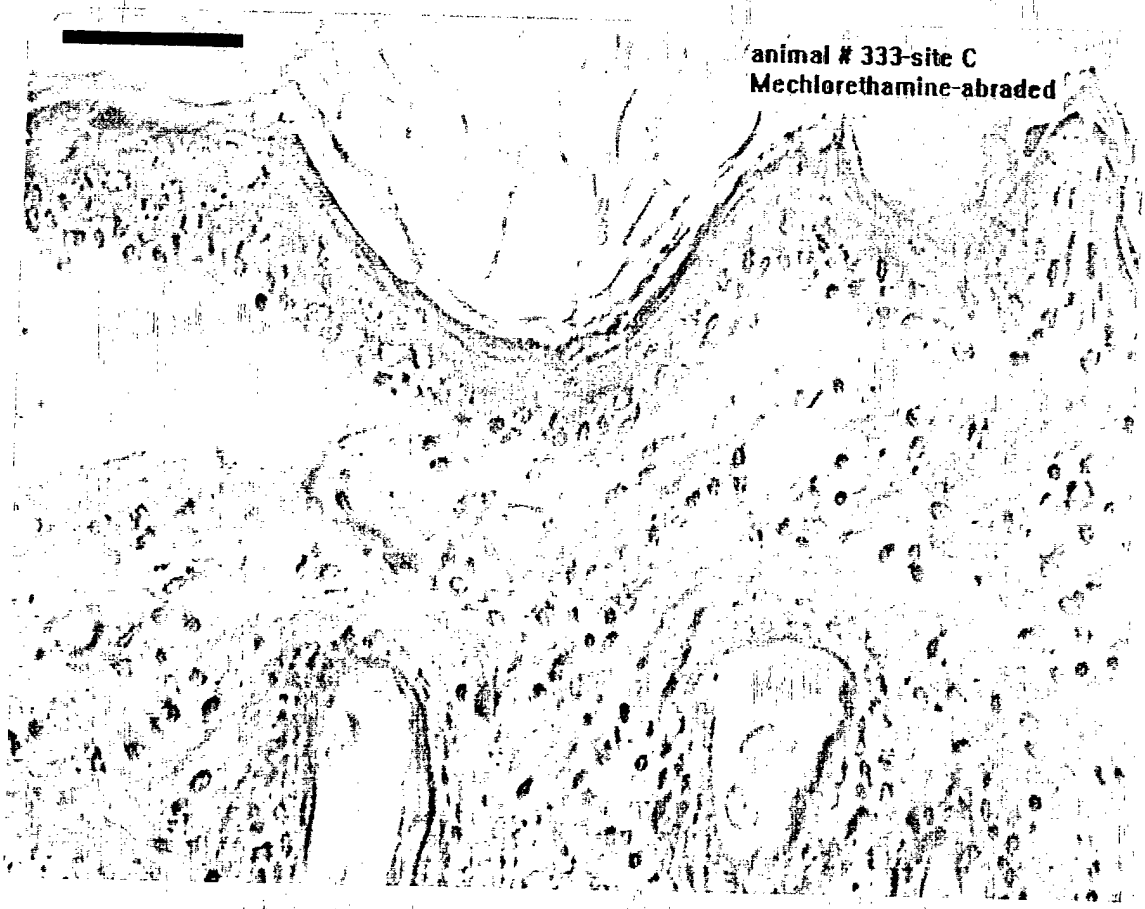

animal # 3188-site F
HD - non-abraded

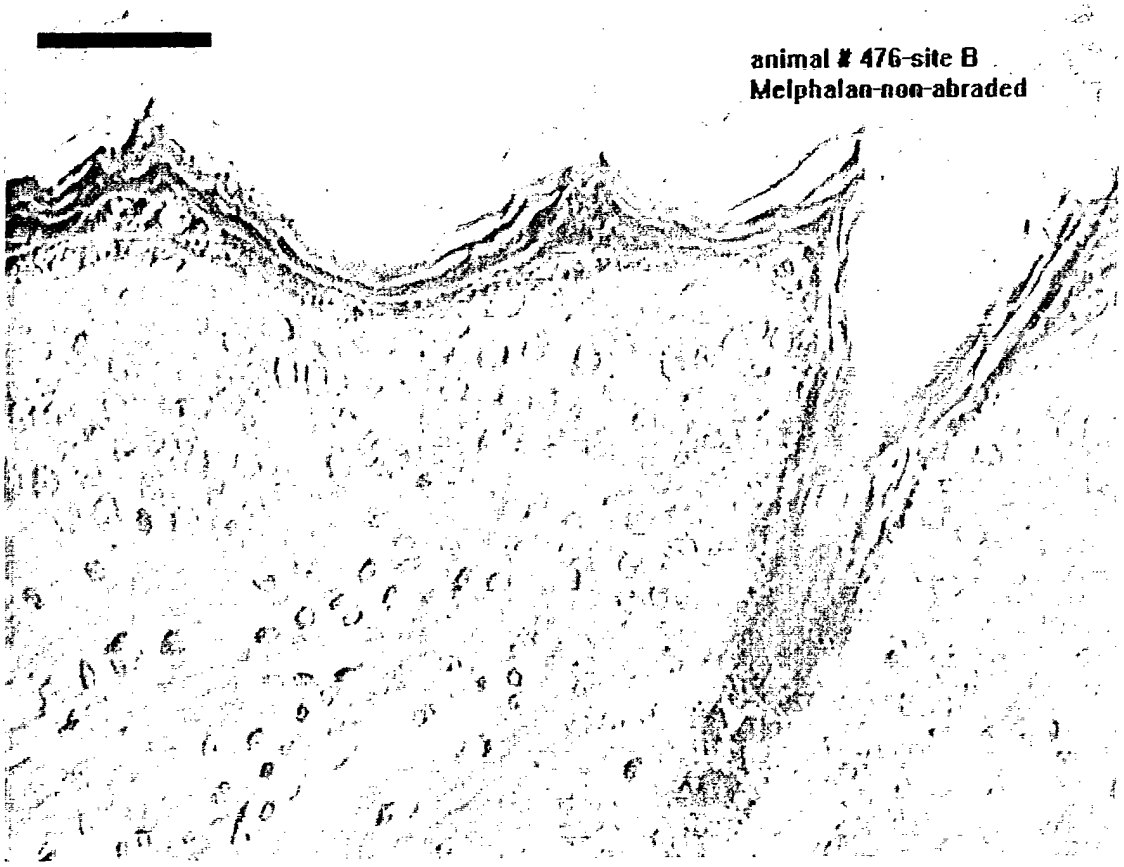

animal 326-site C
RPMI - abraded

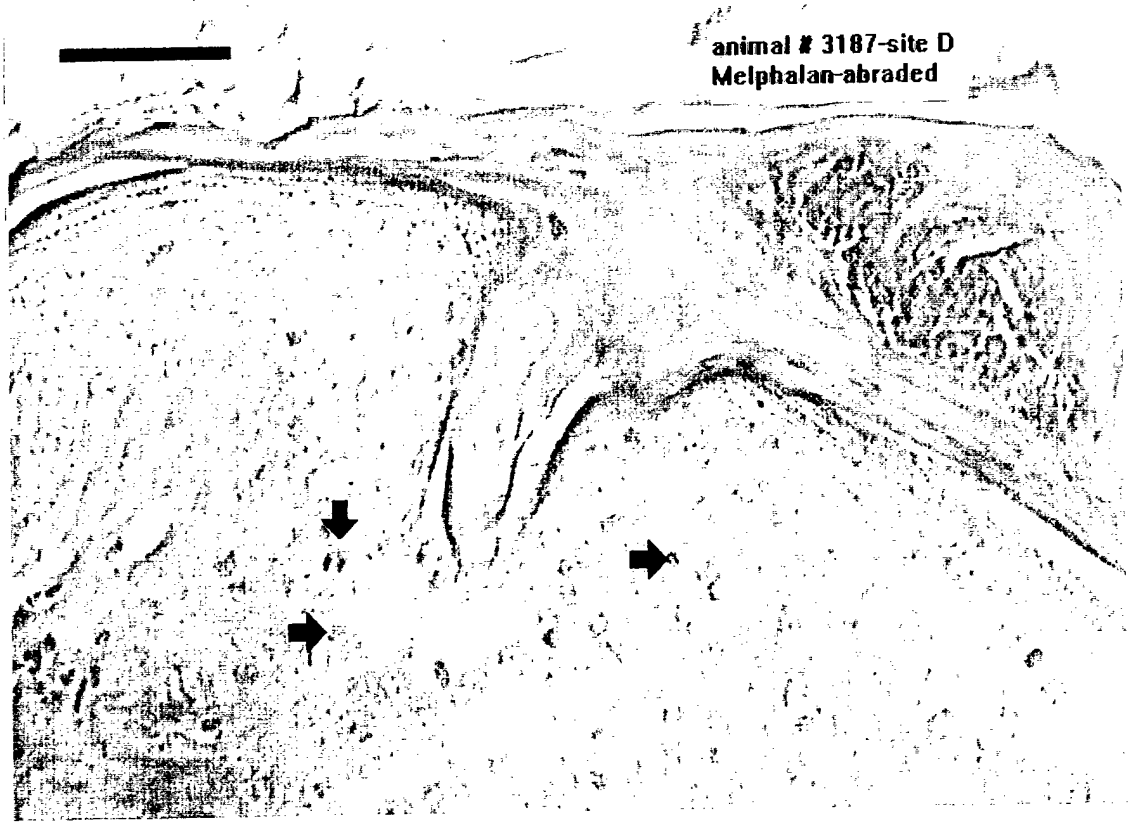

animal # 476-site C
Ethanol - non-abraded

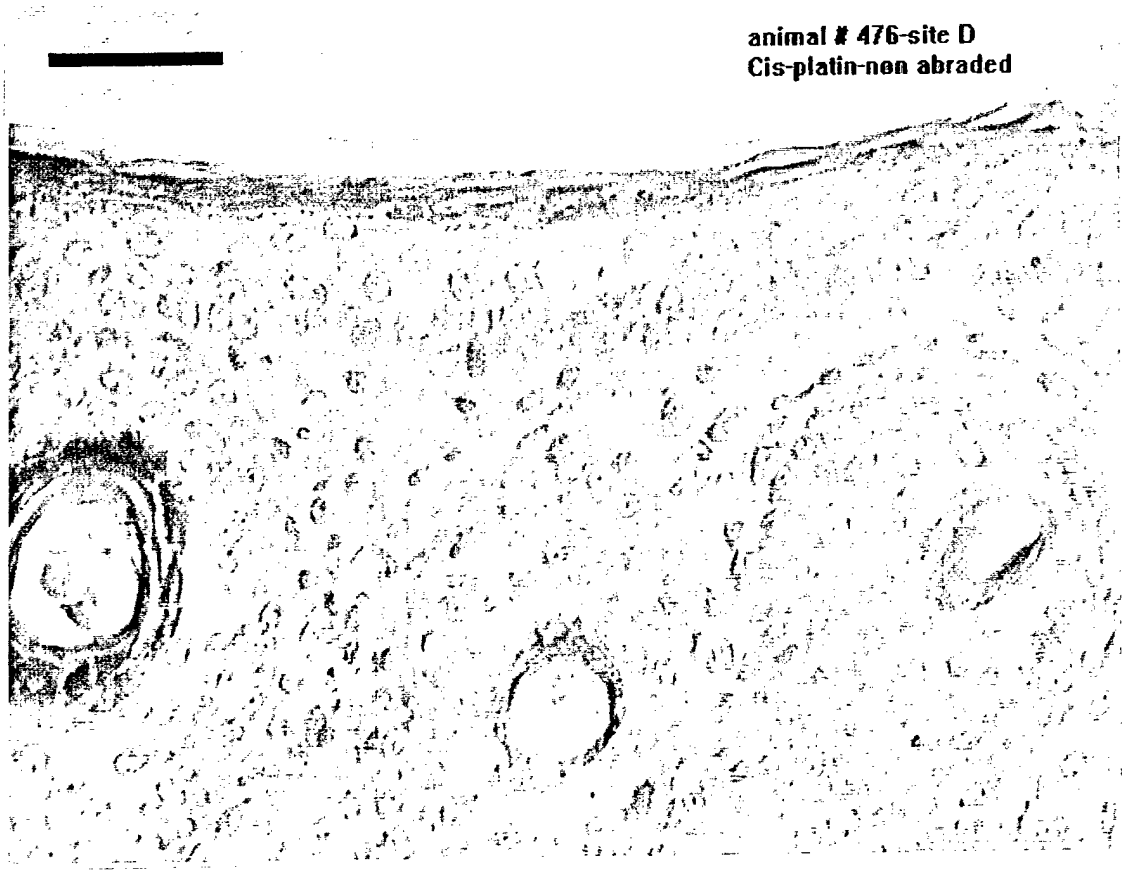

animal # 335-site G
Ethanol - abraded

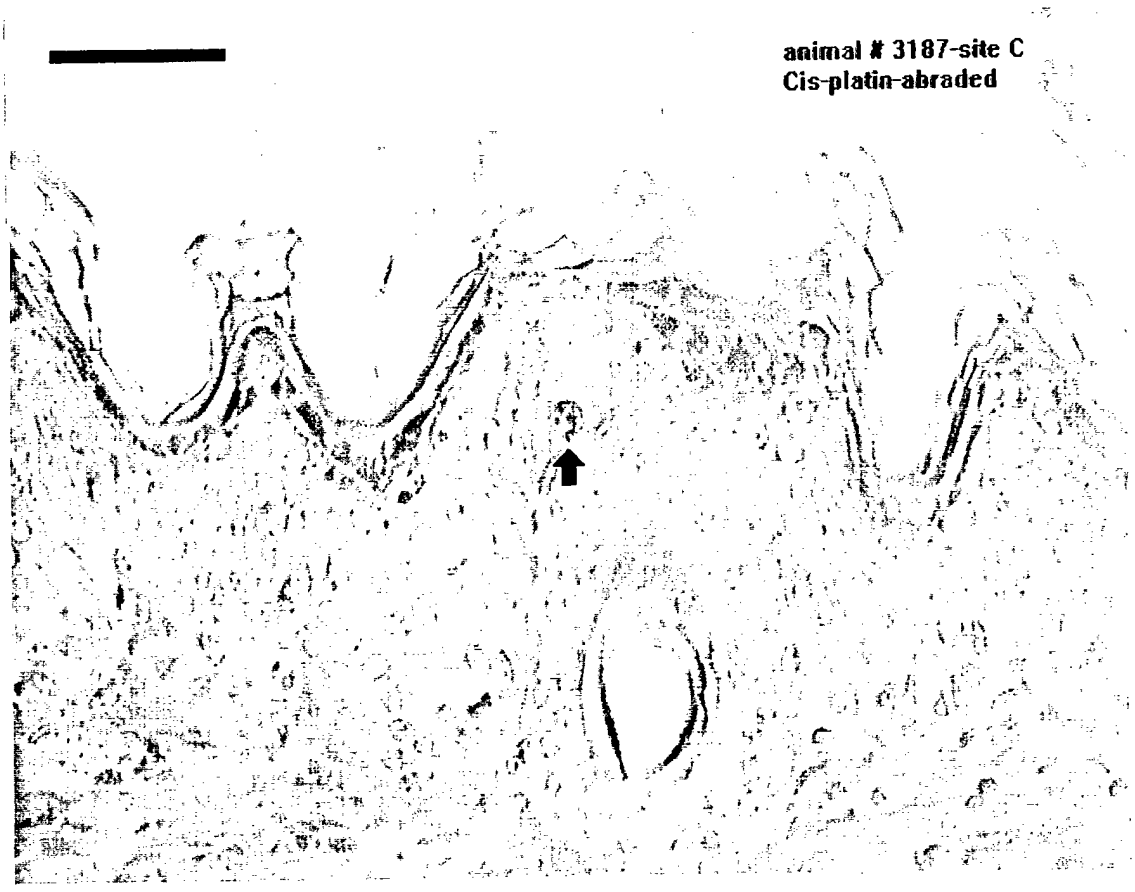

animal # 3183-site B
Mechlorethamine
non-abraded

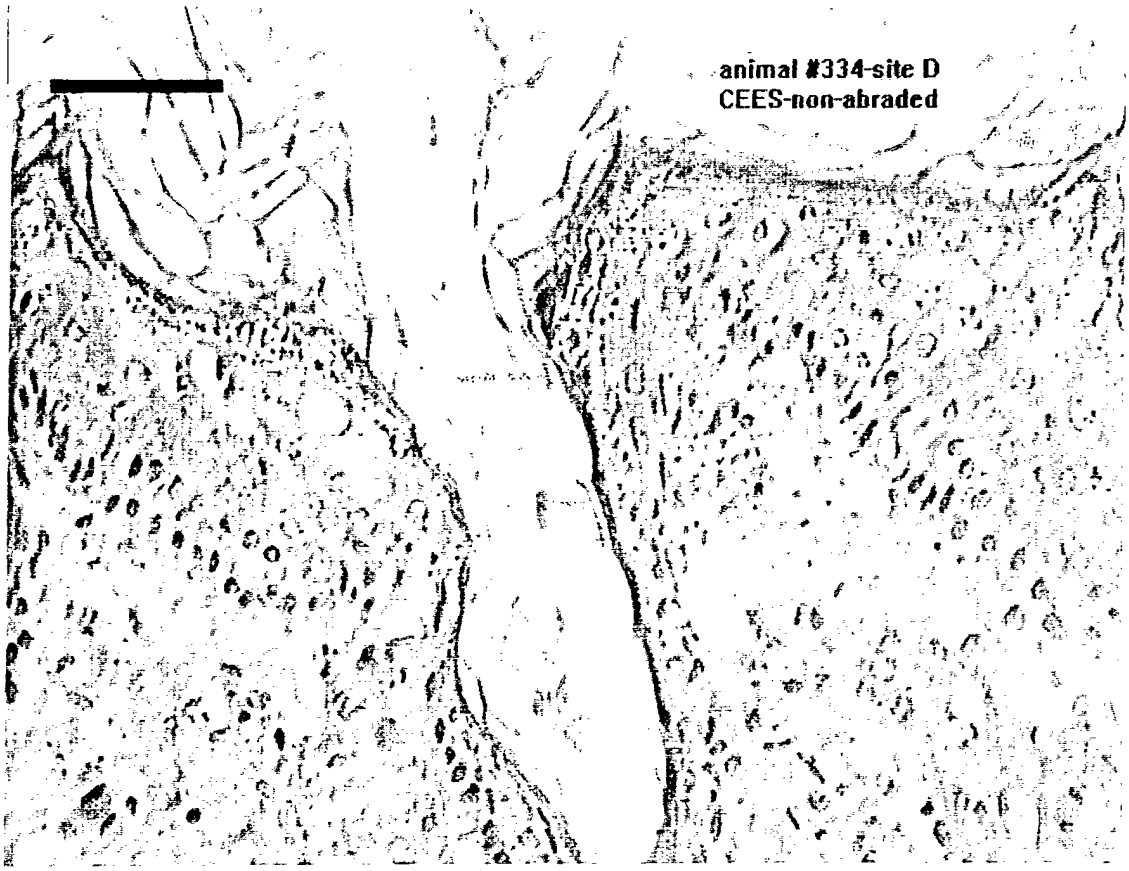

animal # 333-site C
Mechlorethamine-abraded

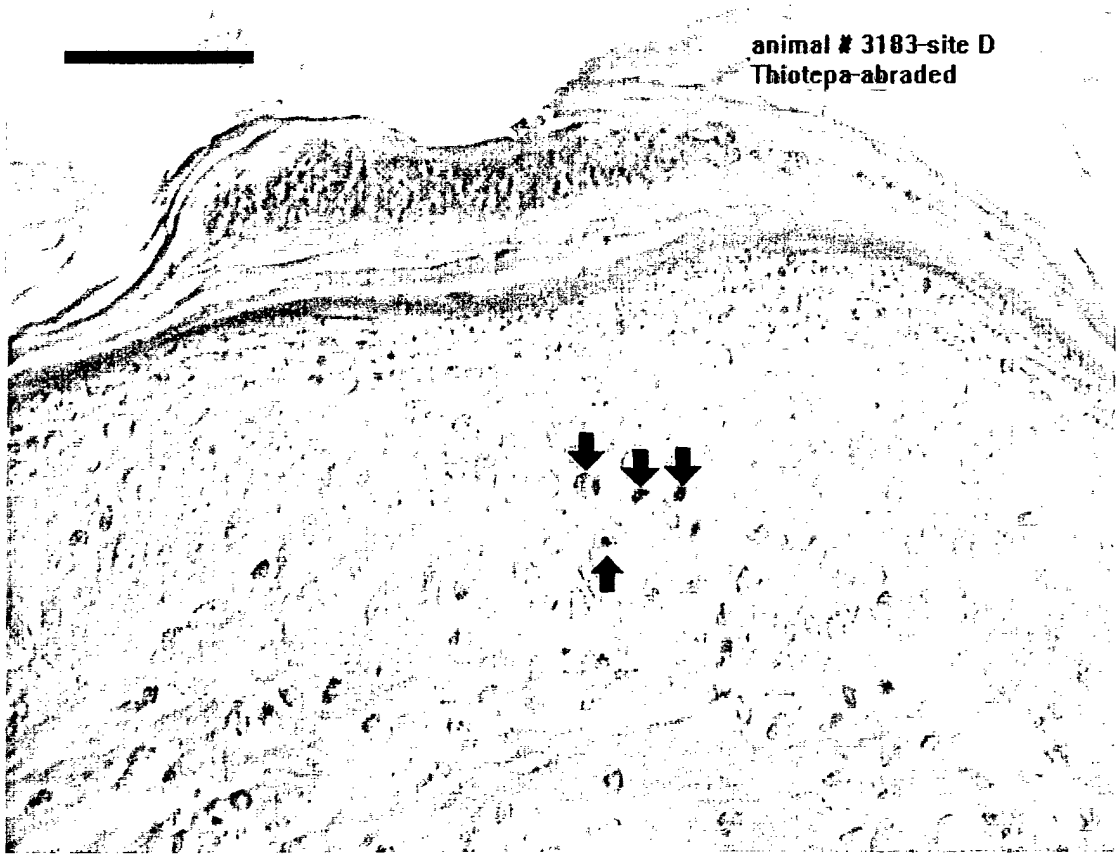

animal # 476-site B
Melphalan-non-abraded


animal # 3187-site D
Melphalan-abraded


animal # 476-site D
Cis-platin-non abraded


animal # 3187-site C
Cis-platin-abraded


animal #334-site D
CEES-non-abraded


animal # 3183-site D
Thiotepa-abraded